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ERRATA

Vol. 18, page 477, line 2 in SUMMARY, "2.9" should read "2.6"
Vol. 19, page 104, line 9 in LITERATURE CITED, "Chargoff" should read "Chargaff"
Vol. 19, page 154, in lines 18 and 23, "A*p*" should read "Aρ"
Vol. 19, page 172, line 15, "alkylate" should read "react with"
Vol. 19, page 185, line 21, "fraction S, P_1" should read "fractions P_1"
Vol. 19, page 232, line 14 should read "The Dowex 50-×8, 200–400 mesh, H^+, was . . ."

Vol. 19, page 237, Table I, last column, "Vaccum" should read "Vacuum"

Vol. 19, page 246, lines 15 and 14 from bottom should read "Dowex 50-×8, 200–400 mesh, H^+, bedded . . ."

Vol. 19, page 247, line 10 from bottom should read "Dowex 50-×8, 200–400 mesh, H^+, resin . . ."

Vol. 19, page 247, line 9 from bottom should read "IR 45-×3.5, 16–50 mesh, OH^+, resin . . ."

Vol. 19, page 283, line 3 from bottom, "*hOO*, *OkO* and *hkO*" should read "*h*OO, O*k*O and *hk*O"

Vol. 19, page 292, line 7, "Captan (N-trichloromethyl mercapto-4-cyclohexene-1,2-dicarboximide)" should read "*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide"

Vol. 19, page 303, lines 15 and 16 of SUMMARY, sentence reading "Of the species with smaller mean spores, i.e., less than 500 μ, 3 tend to . . ." should read "The species with smaller mean spores, i.e., less than 500 μ^3, tend to . . ."

Vol. 19, page 349, add footnote to title as follows: "[1] This work was supported by a grant from the Rockefeller Foundation."

Vol. 19, page 395, line 10 from bottom, "DL-cystine-S^{35}" should read "DL-Cystine-S^{35}. . ."

Vol. 19, page 452, in running title, "Thonpson" should read "Thompson"

Vol. 19, page 460, in running title, "Thonpson" should read "Thompson"

CHANGES IN THE ORGANIC ACID COMPOSITION OF WHEAT LEAVES INFECTED WITH THE LEAF RUST FUNGUS[1]

Richard C. Staples

SUMMARY

A comparison was made of the changes in dry weight, respiratory activity and organic acid content of healthy and leaf-rust infected leaves of Thorne wheat in the period 8 to 23 days after the seeds were sown while uredinial pustules were developing. First true leaves, inoculated with race 9 of *Puccinia rubigo-vera* (DC.) Wint. f. sp. *tritici* (Eriks.) Carleton, were heavier and took up more oxygen than comparable rust-free leaves during the terminal period of infection.

The concentration of malate increased in both rust-free and infected leaves during the period 14 to 23 days while aconitate decreased. These changes appeared to be enhanced by infection but the differences were relatively small (20 to 30 per cent). The citrate content of infected leaves, however, increased substantially while it changed very little in the controls. The reason for these changes in organic acids is not immediately clear but some of the possibilities are discussed.

INTRODUCTION

The obligately parasitic fungi such as the rusts are difficult to study physiologically because they cannot be cultured apart from host tissue. Studies of their pathogenesis have been restricted, as a general rule, to comparisons of healthy and infected tissues and infectivity on different hosts (see e.g., 5). Such information is not sufficient to permit the development of sound hypotheses on the nature of their parasitic processes.

The physiology of the host would be expected to be changed upon establishment of the parasite. Thus, the dry weight of leaves infected with rust fungi apparently increases as a result of the infection (1, 18, 26, 27), although the dry weight of the infected plant as a whole decreases. Changes in pantothenic acid (28) and amino acids (8) have been reported. Radioactive phosphorus, sulfur, and carbon compounds supplied to infected plants have been shown to accumulate most heavily in the vicinity of the rust pustule (9, 20, 21, 29). The infected tissues have a higher respiratory rate (see e.g., 3). Respiration of infected leaves is inhibited less by malonate but more by cyanide than that of healthy tissues (7).

MATERIALS AND METHODS

Three experiments were made. In each, wheat (*Triticum aestivum* L. var. Thorne) was used and inoculation was made with race 9 of *Puccinia rubigo-vera* (DC.) Wint. f. sp. *tritici* (Eriks.) Carleton. (Use of the binomial *Puccinia recondita* Rob. ex Desm. for this fungus has recently been suggested by Cummins and Caldwell [6]). In two experiments the seedlings

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[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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were held in the greenhouse at about 70° to 75° F. during the incubation period for the rust and were harvested in April and in June. In the other experiment the infected seedlings were held in a constant temperature-light room. The temperature of the room was maintained at 70° ± 5° F. when the lights were on and 65° ± 5° F. when the lights were off. The light exposure was eight hours per day. Light intensity of 1200 foot candles with an energy equivalent of 0.45 cal./cm.^{2}/min. was obtained from a combination of Mazda and fluorescent tubes to provide light of a quality approaching that of daylight.

Inoculation of host. Seedlings were grown in a well fertilized garden compost in four-inch pots. About 50 seeds were sown in each of 144 pots and watered twice daily until the seedlings emerged and the first true leaf had attained nearly full length. Inoculation was made on the eighth day.

The seedlings in half of the pots were dusted with a suspension of freshly harvested uredospores in talc diluent. About 0.05 g. of spores were thoroughly mixed in 6 g. of talc and 1 g. aliquots were placed in small paper cups. A cup was then placed in the holder immediately below the vacuum release valve on a large aluminum bell jar that covered 12 pots (13). The jar was then evacuated and the valve opened so the inrushing air dislodged the talc suspension in a cloud over and around the plants. The dusted seedlings were then placed in a high humidity cabinet held at 60° F. (12) for 12 hours. The plants were then either returned to the greenhouse or to the constant light-temperature control room where they were watered twice daily. The inoculated plants were placed in separate groups from the disease-free controls on the same bench so as to avoid contamination. Under the conditions of these experiments the uredinial pustules appeared 6 days later and secondary pustules after 13 to 15 days. At this latter date, 23 days after the seed was sown, both infected and healthy leaves had begun to become chlorotic but had not withered.

Extraction procedures. Leaves were harvested for assay on the thirteenth to fifteenth day after inoculation when the first true leaves were fully infected with an average of about 30 pustules each and the second leaf had only occasional pustules at the tip. Only the first leaves were harvested and these were taken from pots chosen by reference to a table of random numbers to insure uniform sampling. All first leaves in each pot were removed two hours before dawn since the amounts of the various acids in the wheat leaf were found to change at different rates from sunrise to sunset.

Measurements of leaf area showed that 174 leaves gave an adequate sample of population (22). Since 200 leaves would give enough organic acids for analyses this number was used in each sample. Three replicates were harvested on each date and data are presented as an average of the three determinations.

The 200 harvested leaves were placed immediately in 150 ml. of 80 per cent ethanol and ground with a VirTis "45" Homogenizer[2] operating at top speed for two minutes. Where extraction could not be completed immediately the alcoholic suspension of plant brei was stored at −25° C. until needed. The plant solids were removed by centrifugation. The solids were resuspended in 75 ml. of 80 per cent ethanol, stirred and centrifuged until five extractions had been made. The extracts were then combined and elution-analysis begun. Further extraction of the residue by alcohol or by ether after the method of Palmer (16) yielded no detectable acids.

The sorption step. The alcohol extract was filtered through an ion exchange resin according to a modification of the method of Palmer (16). Dowex 1-X10 anion exchange resin (200–400 mesh) was prepared in the formate form and suspended in absolute ethanol. A column 15 × 1 cm. was packed under slight pressure and a plug of glass wool was placed at the top of the resin bed to retain the carotinoids and other constituents which otherwise would clog the top of the column by forming a gum.

The extract was placed in a reservoir flask made from a 500-ml. round-bottom flask equipped with a bottom tube terminating in a 12/30 male joint that would fit into the top of the column. Pressure was then applied to the top of the reservoir flask to give an effluent flow rate of 2 to 3 ml./min. After all the liquid had run through the column, the loading flask and column were rinsed twice with 80 per cent ethanol and then with 50 ml. water. The column was ready for gradient elution.

Separation and identification of acids. Formic acid was passed through the column in continuous gradient elution. A solution of $6N$ acid was forced at a rate of 0.5 ml. per minute into a flask containing 250 ml. of distilled water where it was mixed by a magnetic stirrer and displaced an equal volume of liquid into the resin column in progressively increasing concentration of formic acid.

The eluate was collected in 2-ml. aliquots in test tubes on a fraction collector. The eluates were dried in a water bath at 45° C. under a stream of air to remove the formic acid. The residue was resuspended in 1 ml. CO_2-free water and titrated with $0.01N$ NaOH.

In order to standardize the equipment and provide a quantitative basis for judging the titration data, several tests were made with synthetic mixtures of organic acids. The order of appearance of the acid peaks in the eluate is illustrated in Figure 1. The recovery of acids from the column was exceptionally good. In an experiment where the procedures outlined above were followed, the recovery of succinic acid was 102 per cent, malic acid 99 per cent, citric acid 98 per cent, and aconitic acid 57 per cent. These recovery values were duplicated when known quantities of each acid were added to ground wheat leaves in 80 per cent ethanol. All data present in this paper have been corrected to 100 per cent recovery based on the data of this paragraph.

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[2] The VirTis Company, Inc., Yonkers, New York.

Identity of the acids. The identities of the various acids were first tentatively determined by paper partition chromatography. After titration of the eluates from the resin column, the tubes containing the acids from one peak were pooled and stirred with a cation exchange resin (Dowex 50-X4, 20–50 mesh) in the hydrogen form to remove sodium ions which would have interfered with their paper chromatographic separation. The resin was removed by filtration and the acid solution dried under a stream of air. The acid residue was then taken up in a small amount of water (*ca.* 0.5 ml.) and spotted along the lower margin of six sheets of Whatman No. 1 filter paper cut 25×40 cm. Authentic acids were then pipetted onto the same spots (and alongside to serve as markers). The sheets were stapled into cylinders and each sheet was placed in a different solvent. The six solvents used were the A, D, E and F solvents of Stark, Goodban and Owens (23), the solvent of Resnik, Lee and Powell (17), and Palmer's solvent (16). Solvents were permitted to ascend the sheets and the papers were dried and sprayed with bromphenol-blue indicator. The appearance of acids with Rf values identical to those of the authentic samples in each of the six solvents and the absence of separation of the mixture of authentic and unknown

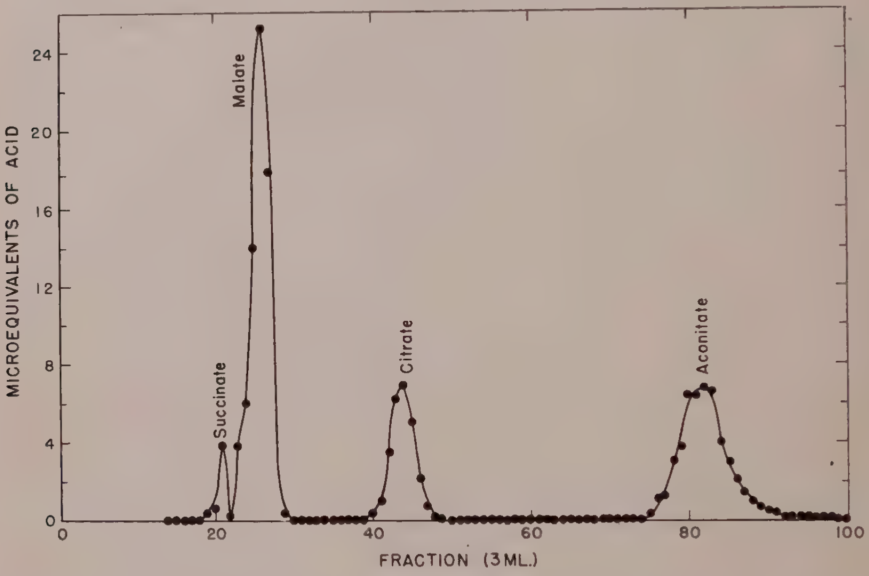

FIGURE 1. Separation of a mixture of authentic acids from a column of Dowex 1 resin by gradient elution with formic acid. Acids were added to the column in 80 per cent ethanol. Conditions of elution and titration were those of Palmer (16).

acids was considered as evidence that the acid had been tentatively identified.

The eluates from various lots of leaves and containing the same acid (on the basis of paper chromatography) were pooled regardless of whether they came from healthy or infected plants and treated with 0.1 g. of Norit-A to remove the indicator used in titration. The Norit-A was removed by filtration and if the filtrate was still colored the Norit-A treatment was repeated. Ten milligrams of Norit-A removed approximately 0.1 mg. of methyl red and 2 per cent of the citric acid. Use of 100 mg. removed 20 per cent, and 500 mg. removed 60 per cent of the citric acid.

The decolorized filtrate was passed through a column of Dowex 50-X4 (20–50 mesh) ion exchange resin to remove sodium ions. The column also served to remove small amounts of suspended matter such as charcoal which may have passed through the filter paper. The water was then evaporated by passing a filtered stream of air over the sample. The acid residue was recrystallized from ether. Fumaric acid was further purified after recrystallization by sublimation at 220° C.

The identity of the crystals obtained from the plants was established by having carbon and hydrogen analyses made by the Schwarzkopf Microanalytical Laboratory (Woodside 77, N. Y.) on 2 to 3 mg. samples. In addition infrared absorption curves were prepared from pellets containing 0.6 mg. of acid in 200 mg. of KBr. Melting points of the crystals were determined by conventional methods except for fumaric acid which was sealed in a tube.

Silica gel chromatography. It was found by paper chromatography that the citric peak contained malonic acid. This malonic acid contaminant had to be removed before citric acid could be isolated. It was also important to determine if the amount of malonic acid present was sufficient to require routine determination of citric acid by different methods. The tubes containing the acids of the citric peak were pooled, dried under a stream of air and the residue taken up in silica gel after the method of Bulen, Varner, and Burrell (4). This silica gel-organic acid mixture was then placed on a silica gel column with chloroform and eluted with a *n*-butanol-chloroform mixture (35:65 v/v). Eluates were collected in 3-ml. fractions. The butanol-chloroform mixture was removed with a stream of filtered air and the acids titrated with 0.010*N* NaOH. Citric acid was then treated further with Norit-A and recrystallized from ether. Malonic acid could not be accurately titrated because the amount was low while the blank was high. Separation of malonate and citrate by the column could be followed by paper chromatography, however, and good separation was obtained. Malonic acid was not isolated from leaves of wheat.

Citric acid determination. Because the amount of malonic acid was low, it could not be estimated by silica gel chromatography. Furthermore, when the wheat leaf was ten days old, the amount decreased, and detection of malonic acid by paper chromatography was difficult. The conclusions of this paper are based upon data obtained after that time so that citric acid was not determined separately.

Respiration of whole leaves. Respiratory activity of wheat leaves was determined by Warburg manometry at 30° C. using a modification of a method by Klinker (10). Five leaves were chosen randomly from the plants for each sample and determinations made in triplicate. The bottom of the Warburg vessel was covered with powdered cellulose and moistened with 0.05 ml. distilled water. Whole leaves were placed in the flask, 0.2 ml. of 10 per cent KOH pipetted into the center well and fluted filter paper added, the flask placed on the manometer and wrapped with aluminum foil. After thirty minutes equilibration, readings were made over a sixty-minute period. At the end of the experiment, the leaves were removed from the flasks and dried to constant weight in an oven at 110° C. for dry weight determinations.

Dry weight determinations. To compare dry weights of healthy and rust-infected leaves, 200 first leaves of Thorne wheat were harvested in triplicate from healthy and infected plants, dried to constant weight in an oven at 110° C. and weighed to the nearest milligram.

RESULTS

SOME EFFECTS OF INFECTION ON THE PHYSIOLOGY OF THE LEAF

The oxygen consumption by rust-free and infected leaves was also determined. The data of Figure 2 A and B show that respiratory activity of the infected leaves increased progressively so that by the end of the experiment it was twice that of the rust-free tissue. This observation holds if the data are expressed on rate of oxygen uptake per unit weight of tissue or number of leaves. These differences were found to be highly significant (22) at the 1 per cent level at the end of the experiment.

The changes in dry weight of the infected and healthy first leaves were determined at three-day intervals during the fortnight after inoculation. There was no appreciable change during the first five days as shown by the data in Figure 2 C. However, beginning with the sixth day the leaves from rust-free plants began to lose weight while those from the infected plants lost less weight and even gained during the last six days. The difference between the mean dry weights of the healthy and infected leaves, at the end of the experiments, was highly significant at the 1 per cent level. Infection resulted in a 22 per cent increase in the dry weight of the leaf.

ORGANIC ACID COMPOSITION OF THE FIRST WHEAT LEAF

Succinic, malic, citric, fumaric, and aconitic acids were isolated from the first leaf of wheat. Crystals of these isolates were characterized by phys-

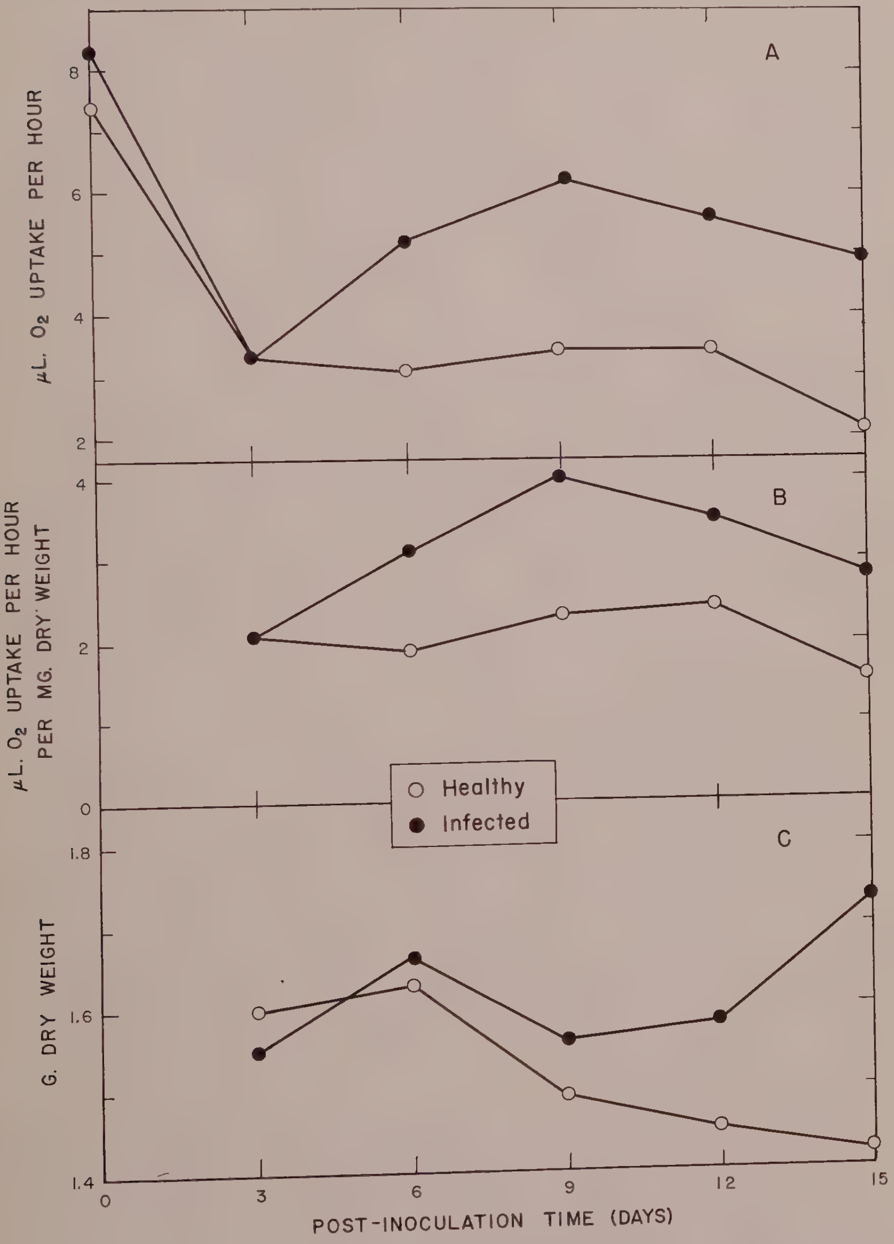

FIGURE 2. Oxygen uptake and changes in dry weight of whole leaves of Thorne wheat. Infected leaves were inoculated with leaf rust uredospores when the seedlings were 8 days old. Oxygen uptake is expressed as μl. O_2 per hour. (A) μl. O_2 per hour per 200 leaves; (B) μl. O_2 per hour per mg. dry weight; (C) g. dry weight per 200 leaves.

TABLE I

MELTING POINTS OF CRYSTALS OBTAINED FROM WHEAT LEAVES COMPARED TO THOSE OF AUTHENTIC SAMPLES OF ORGANIC ACIDS

Acid	Melting points (° C.) (corrected)		
	Known	Unknown	Mixed
Succinic	184–185	184 –185	184–185
Malic	102–103	100 –101	100–101
Citric	151–153	148 –150	148–152
Fumaric	285–286	281.5–283	—
trans-Aconitic	168–169	173 –173.5	172–174

ical methods described above. There were no observed depressions of mixed melting points (Table I). Infrared absorption curves of the crystals corresponded exactly with those of the known samples (Fig. 3). Carbon and hydrogen analyses of the crystals gave percentages of carbon and hydrogen which were close to values calculated for these acids (Table II). Malonic acid was not isolated but was tentatively identified by co-chromatography in six solvents as described in the previous section.

The melting point of aconitic acid indicated a possible mixture of *cis*- and *trans*-isomers, since its melting point was lower than that of pure *trans*-aconitic acid. Mixtures of *trans*- and *cis*-aconitic acids have melting points intermediate between those of the pure *cis*- and *trans*-acids (14). The unknown was shown to be a mixture of these isomers by paper chromatography, using solvent F of Stark, Goodban and Owens (23).

Oxalic acid, reported in wheat plants by Nelson and Hasselbring (15), was not found, possibly because it is not eluted from the anion exchange column under the conditions employed in these experiments (16).

The amounts of the organic acids in the first leaf of wheat were found to vary with age of the leaf. Values found for the various acids are listed in Table III under the column labeled "Healthy".

Changes in the quantities of the nonvolatile organic acids were followed from dawn until sunset (Fig. 4). These studies were carried out on leaves 13 days old. It was found that malic acid increased rapidly after sunrise,

TABLE II

RESULTS OF CARBON-HYDROGEN ANALYSES OF CRYSTALS OBTAINED FROM WHEAT LEAVES (% DRY WEIGHT)

Acid	Theory		Found	
	C	H	C	H
Succinic	40.7	5.1	40.8	5.2
Malic	35.8	4.5	36.1	4.5
Citric	37.5	4.2	37.7	4.1
trans-Aconitic	41.4	3.5	41.5	3.7

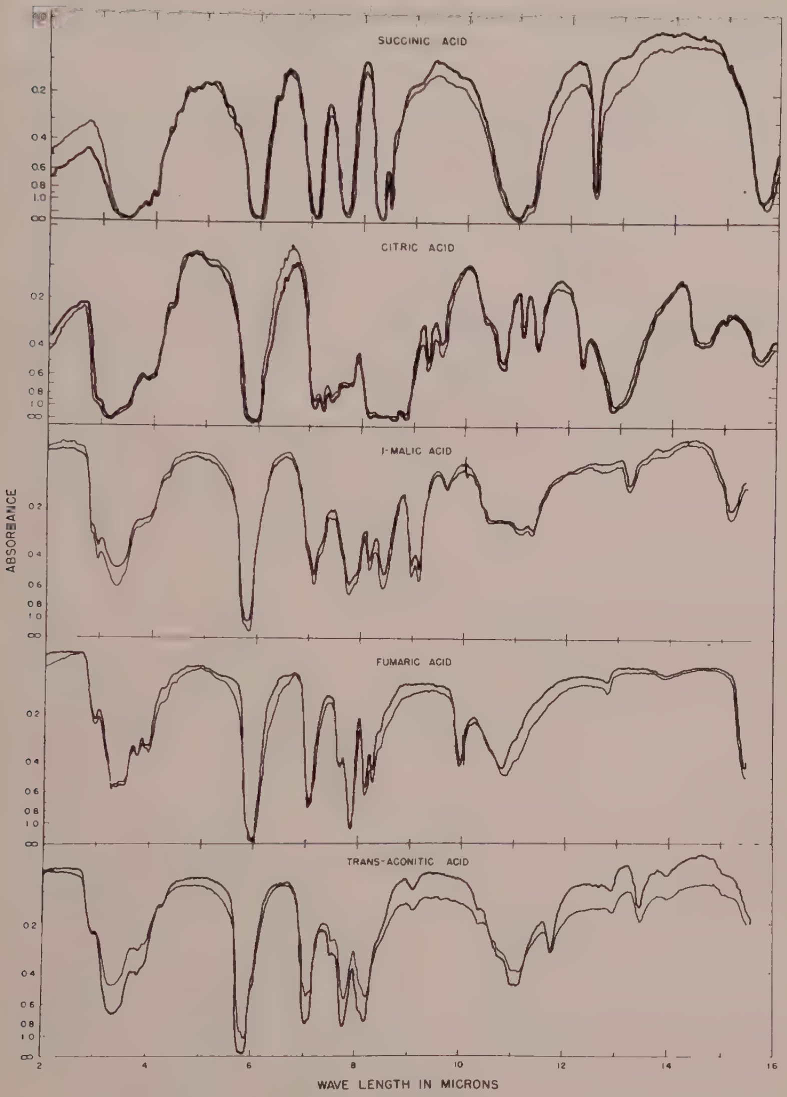

FIGURE 3. Comparison of infrared absorption curves of crystals isolated from wheat leaves (light line) and authentic samples of corresponding organic acids (heavier line).

TABLE III

QUANTITATIVE CHANGES IN ORGANIC ACIDS OF THE FIRST LEAF OF WHEAT. VALUES ARE ADJUSTED TO 100% RECOVERY (SEE TEXT)

Age of leaf (days)	Post-inoculation time (days)	Milliequivalents of acid per 200 leaves from					
		Greenhouse-April		Greenhouse-June		Light chamber	
		Inoculated	Healthy	Inoculated	Healthy	Inoculated	Healthy
		Malic acid					
11	3	0.094	0.085	0.079	0.127	—	—
14	6	0.036	0.024	0.267	0.119	0.075	0.064
17	9	0.259	0.258	0.652	0.492	—	—
20	12	1.000	0.909	0.851	0.711	0.139	0.103
23	15	1.173	0.816	0.896	0.702	—	—
		Citric acid					
11	3	0.102	0.110	0.076	0.065	—	—
14	6	0.033	0.038	0.054	0.041	0.047	0.036
17	9	0.072	0.055	0.106	0.050	—	—
20	12	0.074	0.044	0.188	0.044	0.096	0.056
23	15	0.138	0.065	0.211	0.048	—	—
		Aconitic acid					
11	3	0.408	0.350	0.412	0.438	—	—
14	6	0.242	0.317	0.280	0.301	0.127	0.172
17	9	0.180	0.239	0.127	0.291	—	—
20	12	—	—	0.092	0.247	0.008	0.057
23	15	0.022	0.082	0.033	0.097	—	—
		Fumaric acid					
11	3	0.005	0.003	0.004	0.005	—	—
14	6	0.004	0.004	0.002	0.0003	Trace	Trace
17	9	0.010	0.005	0.005	0.0001	—	—
20	12	0.018	0.008	0.002	0.0006	Trace	Trace
23	15	0.022	0.004	0.004	0.0006	—	—
		Succinic acid					
11	3	0.009	0.012	0.004	0.007	—	—
14	6	0.009	0.006	0.017	0.005	0.005	0.003
17	9	0.016	0.009	0.004	0.0004	—	—
20	12	0.024	0.009	0.003	0.0009	0.007	0.003
23	15	0.021	0.006	0.006	0.0004	—	—

while the amounts of citric and aconitic acids did not change. The amount of succinic acid rose toward the end of the day. Malic and aconitic were the most abundant acids in the leaf. Aconitate concentration was three times that of malate at dawn while malate concentration nearly equalled that of aconitate at sunset. All data in experiments to be described below represent the organic acid composition of leaves harvested at dawn.

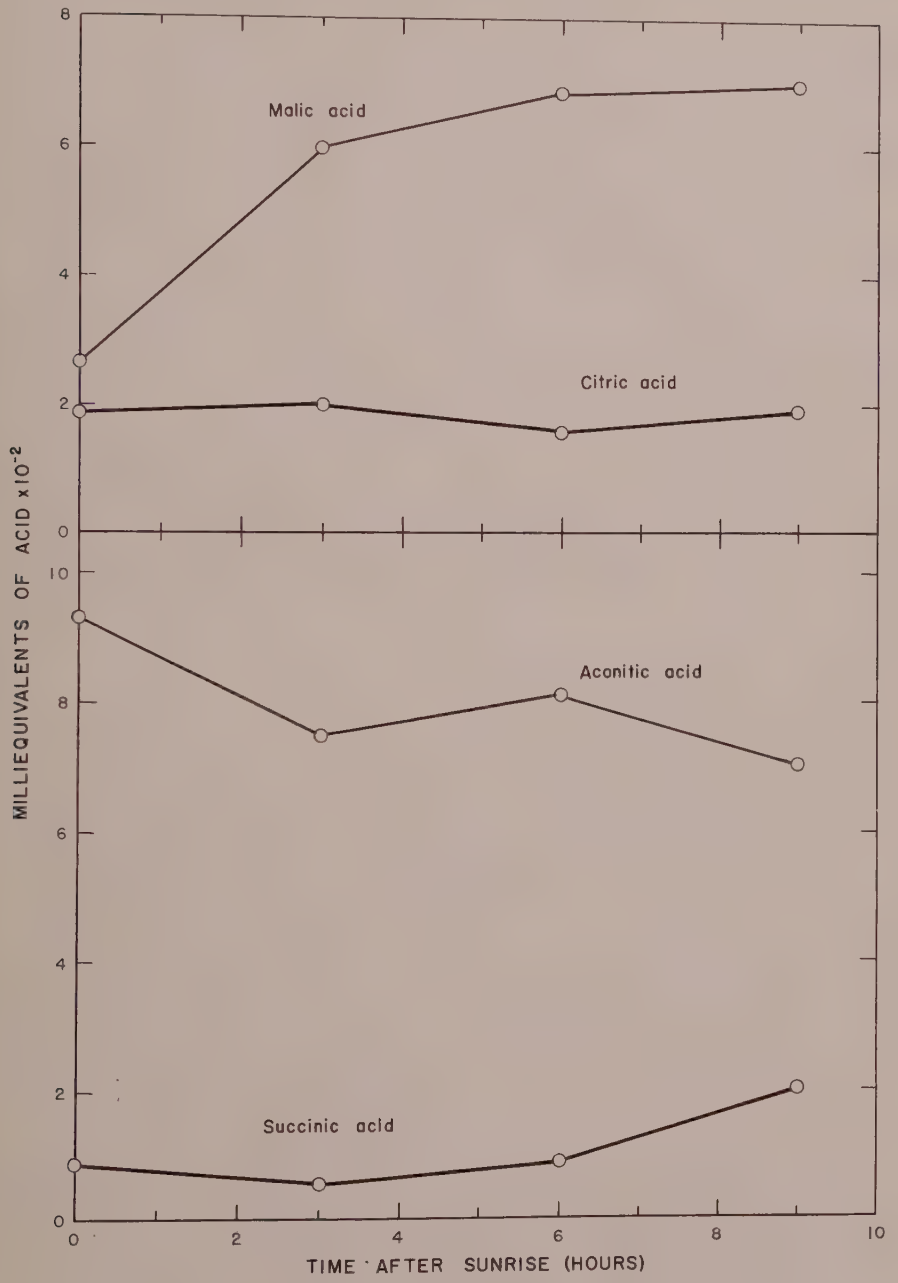

FIGURE 4. Diurnal changes in the organic acid content of wheat seedlings harvested 13 days after planting.

Figure 5 depicts the changes of malic, citric, and aconitic acids as found in the June experiment. Values for all acids found in all experiments are summarized in Table III. Values for fumaric and succinic acids are not depicted graphically because the quantity of these acids was so low that the experimental error of their determination was necessarily high. The leaves in the June experiment were harvested under uniform conditions of light and temperature. The light values are all within the range of 328 to 448 cal./cm.^{2}/day with a coefficient of variation (22) of 12 per cent. This was not the case for the April experiment, in which the light values ranged from 66 to 696 cal./cm.^{2}/day with a coefficient of variation of 41 per cent.

Considering the healthy leaves of the June experiment (Fig. 5), malic accumulated as the leaf aged to such an extent that, of the acids measured, it eventually predominated. This accumulation began after the leaves were 14 days old. The quantity of citric acid remained fairly constant in the healthy leaf and did not change greatly as the leaf aged. Aconitic, the predominant acid when the leaf was younger than 14 days, decreased progressively with age. At the end of this experiment, malic acid had increased sevenfold while aconitic acid had decreased about fivefold. Citric acid remained rather low throughout the period of the experiment. Similar changes occurred in the other experiments (Table III).

ORGANIC ACID COMPOSITION OF INFECTED WHEAT LEAVES

The same nonvolatile organic acids were found in the infected wheat leaf (Fig. 6 A) as were found in the healthy wheat leaf (Fig. 6 B). Acids from the infected wheat leaf were identified by paper chromatography in six solvents as previously described. These acids, in crude form, were added to the crude acids from the healthy leaves, and the mixture purified. This procedure increased the supply of crystals available for analyses and confirmed identifications made by paper chromatography.

The quantities of the nonvolatile organic acids in infected leaves, for increasing leaf age, are listed in Table III. The changes in the amounts of citric, malic and aconitic acids, as found in the June experiment, are depicted in Figure 5. Malic and citric acids accumulated with leaf age but aconitic acid decreased. Changes in the acids took place about the time of first sporulation (6 days after inoculation of the leaves, 14 days after planting).

Infection of the wheat leaf with the leaf rust fungus did not affect the trends of malic and aconitic acids though, at the end of the June experiment, citrate increased about 400 per cent in the inoculated leaf over that in the control. This increase was progressive following inoculation as depicted in Figure 5. Even in the inoculated leaf, however, the total level of citric acid was considerably below that of malic acid (Table III) at the end of the experiment.

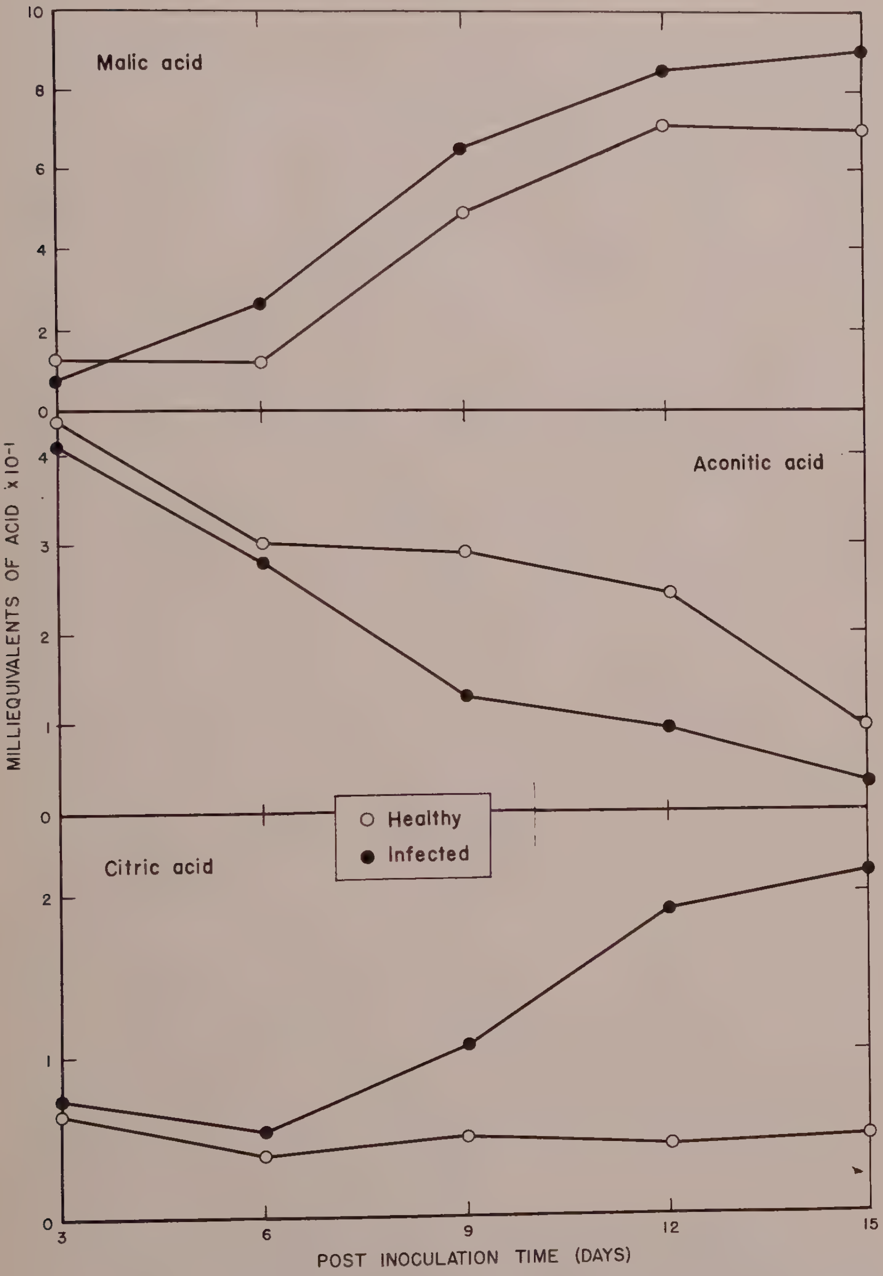

FIGURE 5. Quantitative changes in organic acids of first wheat leaf. Infected leaves inoculated 8 days after planting. Harvest at dawn. Data of June experiment, Table III.

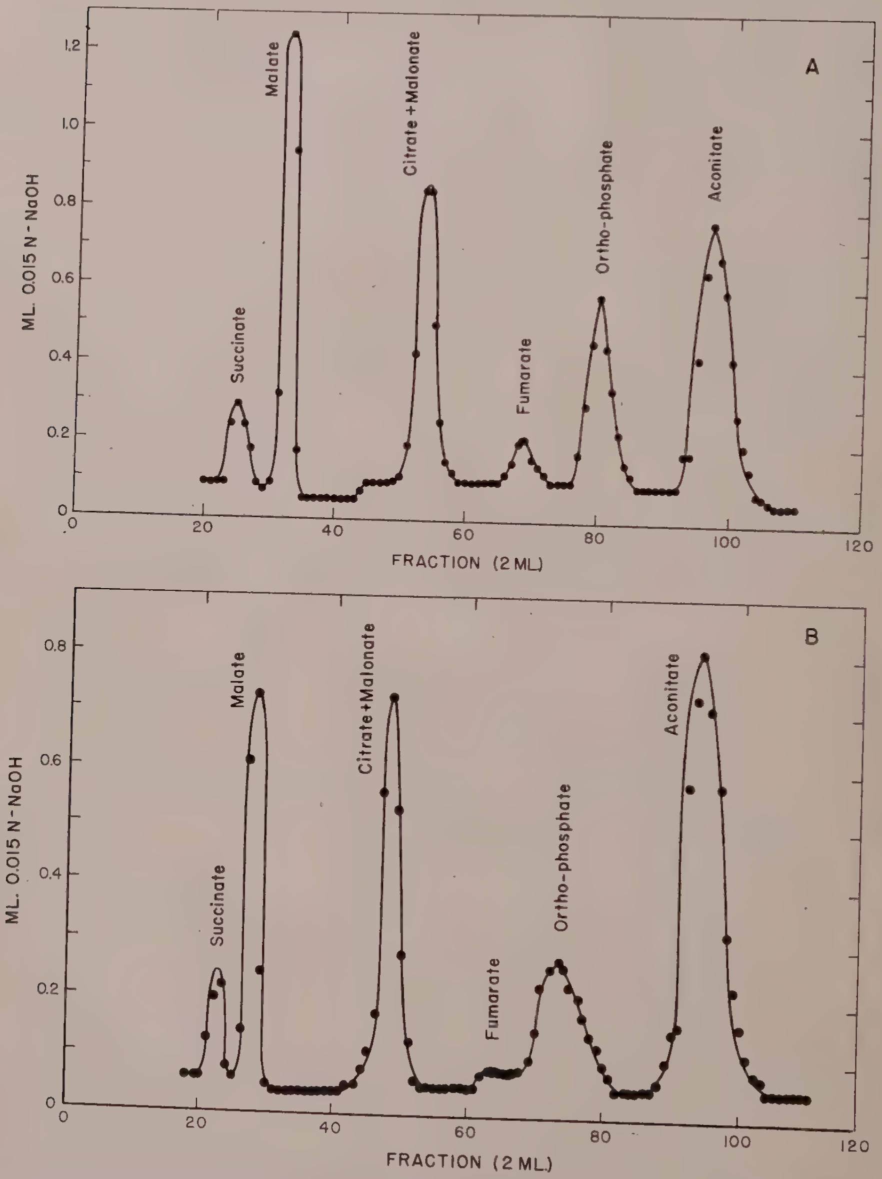

FIGURE 6. Comparison of organic acid content of 80 per cent ethanol extract of infected (A) and rust-free (B) leaves of Thorne wheat seedlings six days after inoculation. Curves obtained by titration of fractions of the eluate with standard base after removal of formic acid.

In the June experiment, malic acid increased about sixfold in healthy leaves (Fig. 5), and about tenfold in the inoculated leaves. Malic acid was about 22 per cent higher in the infected leaves than in the controls at the end of the experiment. Aconitic acid decreased about fourfold in the healthy leaves, at 15 days after inoculation, decreasing twelvefold in the inoculated leaves. Thus, aconitic acid was 30 per cent lower in the inoculated than in the control leaves at the end of the experiment. Similar changes took place in other experiments (Table III).

Again in the June experiment, citric acid changed very little in healthy leaves during the course of the experiment, but increased about fourfold in infected leaves. This was a direct departure from the trend of the acid in healthy leaves. Similar changes took place in the other experiments (Table III). The rapid rise of citric acid in the infected wheat leaf appeared as an exception to the general behavior of the other acids.

DISCUSSION

The acids of the Krebs tricarboxylic acid cycle occupy a central role in the metabolism of the cell. Changes in the quantities of Krebs cycle acids may reflect changes in the quantities of many substances the metabolism of which is linked directly or indirectly to the tricarboxylic acid cycle. Thus, changes in tricarboxylic acid cycle intermediates should be useful as an indication of certain kinds of alterations in the metabolism of the host-parasite complex. Variations in the amounts of these acids occur as the healthy wheat leaf ages. By comparing these variations with those occurring in the diseased leaf during the course of the infection, it was hoped to infer something of the nature of the processes taking place in the diseased leaf and whether they are due to the activity of the fungus or to the metabolism of the host as affected by the fungus.

After the healthy wheat leaf was older than about 11 days, the leaf entered a period of senescence which was characterized by continual decreases in respiratory rate (Fig. 2 A, B) and in dry weight (Fig. 5); a progressive increase in malate; and a decline in aconitate (Fig. 5). Citrate did not change during this senescent period. From the time the leaf was inoculated (8 days after planting), and until the fungus had begun sporulation six days later, there was little difference between the infected and healthy leaves in terms of dry weight (Fig. 5). The rate of oxygen uptake (Fig. 2 A, B) began to rise after about 3 days of incubation with the rust fungus. After the time of first sporulation by the fungus both the rate of oxygen uptake and the dry weight of the infected leaves were increased.

The host-parasite complex (19), acting as Walkley conceived a metabolic sink (24), apparently reversed the outward flow of the translocated-materials typical of the normal aging wheat leaf (25). The increase in oxygen uptake of the infected leaf indicated an increased rate of metabolism, which would fulfill Walkley's concept of a sink.

The cause of the reversal of the trend of dry weight loss by the leaf as a result of infection (i.e., the identity and nature of the sink within the leaf) is unknown. It may be that leaf constituents are absorbed by the fungus without a great disturbance of the host cells. Materials are absorbed from the leaf by the fungus (18), but the greater importance of the leaf cells as the cause of this accumulation has been suggested by Allen (2) and more recently by Shaw and Samborski (18, 21). Their conclusions were that most of a respiratory increase of the host-parasite complex was due to a fungus-induced increase in the respiration of the host cells. Both investigations demonstrated that this augmented metabolic increase was peculiar to the host-obligate parasite complex (as contrasted to injured leaves, for example, including leaves injured by facultative parasites). The evidence summarized by Allen (2) was mostly for powdery mildew on wheat. This fungus can be separated from the host (except for the haustoria). Shaw and Samborski (21) have compared powdery mildew and stem rust of wheat in their experiments. They present evidence that accumulation of materials in rust- and mildew-infected leaves "is linked with their enhanced aerobic metabolism" (21, p. 402). They also claim that the accumulation, as demonstrated by microautoradiography, takes place in wheat leaf tissue and not in the mycelia of the stem rust fungus. The evidence for both fungi was in favor of this hypothesis, but the evidence for powdery mildew was more conclusive. Yarwood and Jacobson (29) also have obtained evidence in support of the contention that the accumulation of materials takes place in the host tissue and not in the fungus. In their experiments they heated the host-parasite complex (by dipping in water at 45° C. for 100 sec.) so that the fungus but not the host was killed as judged by the inability of the fungus to sporulate and lack of further growth of pustules already formed. Radioactive phosphorus accumulated at the site of such infections, though at a somewhat reduced rate.

All of the nonketo acids of the Krebs citric acid cycle (11), except isocitrate, were isolated from the wheat leaf. Trace amounts of these acids would not have been detected by the methods used, hence the amounts reported may represent estimates of pool sizes. The same complex of acids was isolated from wheat leaves infected with the leaf rust fungus. Thus, infection of the leaf by this parasite did not result in a qualitative change in the organic acid complex of the wheat leaf

In the infected leaf, malate and aconitate underwent essentially the same changes as occurred in the healthy leaf. These changes were enhanced in the host-parasite complex in that malate-increases and aconitate-losses were greater in the infected than in the healthy leaf at the end of the experimental period. The differences were small (*ca.* 20 to 30 per cent) and may have been in response to the enhanced metabolism of the host-parasite complex. As cited above, such a suggestion has been made for the observed increase in the dry weight of wheat leaves infected with the stem rust fungus (21).

Citrate increased abruptly at about the time of first sporulation of the fungus (Fig. 5) and increased fourfold thereafter. This departure of citrate, in infected leaves, from the general pattern of behavior of this acid in healthy leaves is more difficult to explain. Since the pattern of accumulation was materially different only in the case of citrate, it is probable that infection of the leaf did not result in a greatly deranged leaf metabolism. It is possible, for example, that citrate may be a product of the metabolism of the fungus, or that the equilibrium between citrate and its precursors in the leaf cells may be disturbed by the presence of the fungus, so that citrate is formed faster than it can be utilized.

If the views of Shaw and Samborski (18, 21) are correct, the observed changes in dry weight and oxygen uptake occurred mainly in the host tissue. Failure of occurrence of substantial differences in qualitative and quantitative organic acid composition between healthy and infected leaves suggests that there was no significant qualitative or quantitative (except for citrate) alteration in organic acid metabolism resulting from the presence of the parasite. The observed change in citrate agrees closely with the change in rate of oxygen uptake.

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THE ORGANIC ACID COMPOSITION AND SUCCINOXIDASE ACTIVITY OF THE UREDOSPORE OF THE LEAF AND STEM RUST FUNGI[1]

RICHARD C. STAPLES

SUMMARY

The organic acid composition of uredospores of both *Puccinia rubigo-vera* (DC.) Wint. f. sp. *tritici* (Eriks.) Carleton and *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn. have been studied. Both species contained malonate, citrate, succinate, malate, fumarate, aconitate and an unknown acid in quantities sufficient to be detected by paper chromatography. These tentative identifications from papers developed in six different solvent systems were confirmed for malonate and citrate by isolating the crystalline materials and comparing their elemental composition, melting points and infrared absorption spectra to those of authentic samples.

The oxygen uptake of whole uredospores of both species was accentuated by addition of succinate but not by glucose, citrate, malonate, malate or fumarate. The metabolism of succinate did not increase the production of carbon dioxide. The succinate oxidizing system of both species was competitively inhibited by malonate and was sharply inhibited in the leaf rust fungus by cyanide. The acceleration of respiration by succinate was largest over the range of pH 6.0 to 7.0 but declined sharply in more alkaline solutions. Endogenous respiration was most active at pH 6.0 and declined sharply on either side of this optimum.

INTRODUCTION

Uredospores are probably the most useful material for studying the metabolism of the rust fungi because they can be obtained in quantity and will germinate readily on a water surface or water agar. A study of the cell constituents consumed during this growth might very well cast some light on the metabolic processes essential for culturing these fungi apart from their hosts.

The organic acids in the spores and changes in their composition during germination were considered a likely area for investigation. There is very little information available on the composition of the uredospores other than reports on D-glucose, D-mannose and D-arabitol (6, 15) and on certain volatile inhibitors and other components that affect germination (1, 2, 8, 9, 23).

During the germination of uredospores, the respiratory quotient follows the pattern of lipid consumption. Shu, Tanner, and Ledingham (17) found that in addition to lipids, some proteinaceous materials were utilized. Studies were undertaken on the nonvolatile organic acids in the leaf rust

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[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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fungus of wheat (*Puccinia rubigo-vera* f. sp. *tritici*) and were extended in part to race 15B of the stem rust fungus (*Puccinia graminis* f. sp. *tritici*). Data on the composition of uredospores and their respiratory response to exogenously applied substrates are summarized in this report.

MATERIALS AND METHODS

Uredospores of the leaf rust fungus were collected under field conditions. These collections inevitably contained some foreign matter such as conidia of *Alternaria* spp. Careful counts revealed that there was no more than one conidium per thousand uredospores. The dry spores were placed in a tightly stoppered glass bottle and stored at 4° C. Collections were made through the courtesy of collaborators in 1954 and 1955. Uredospores of the stem rust fungus, race 15B, were harvested from wheat grown under greenhouse conditions.

All samples of spores were capable of germinating in excess of 90 per cent when used. Careful microscopical examinations were made at the end of all experiments conducted in buffered solution to ascertain that there was no appreciable population of bacterial or fungal contaminants.

Respiratory measurements were made in Warburg respirometers at 30° C. by standard techniques (21). Usually 35 mg. of uredospores were suspended in 1 ml. of $0.5M$ phosphate buffer or $0.2M$ phthalate buffer by gently rubbing the spores with a glass rod until they were wet and readily suspended. Microscopical examination of the spores failed to reveal any mechanical damage before they were transferred to the main compartment of the flask.

Where oxygen consumption was to be measured, carbon dioxide was absorbed by 0.2 ml. of 10 per cent KOH placed in the center well with a 2-cm. fluted filter paper square. For carbon dioxide determination the three-flask modification of Warburg's direct method was used (21, pp. 17–20). After the system was set up, 1.5 ml. of substrate solution or distilled water was added to the spore suspension in the main compartment. After allowing 10 minutes for equilibration, the test was started. Where inhibitory substances, such as malonate, were to be added, 0.5 ml. of the solution was tipped in from the side arm into sufficient spores and substrate to provide a total volume of 2.5 ml.

Organic acids were extracted from 10.0 g. of whole uredospores by shaking in 80 per cent ethanol for five days. The ethanol extract was removed once a day and another aliquot added until a total of about 500 ml. of extract was obtained. All nonvolatile Krebs tricarboxylic acid cycle intermediates which were detected, except citrate and malonate, were quantitatively extracted by this method (Table I).

For more nearly complete extraction of citrate and malonate a modification of the Palmer method (14) was adopted. The ethanol-extracted

TABLE I

ORGANIC ACID CONTENT OF RESTING UREDOSPORES OF THE LEAF RUST FUNGUS

Acid	Milliequivalent of acid per 10.0 g. spores				
	Alcohol extraction			Reaction with ether	
	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2
Succinic	0.017	0.017	0.015	0	0
Malic	0.017	0.021	0.023	0	0
Citric+malonic	0.211	0.219	0.219	0.834	0.908
Fumaric	0.003	0.003	0.004	0	0
Aconitic	0.007	—	0.011	0	0

spores were dried, stirred with 20 ml. of $0.4N$ sulfuric acid and ether-extracted asbestos until the mass became viscous, and then extracted with ether for five days. The ether was evaporated, the residue treated with barium hydroxide until most of the sulfate, but not quite all had been precipitated, the precipitate was removed by filtration and the extract was placed on an ion exchange column.

The acids in the ethanol or ether extracts were separated by gradient elution with formic acid from a 1×15 cm. column of Dowex 1-X10 (formate form) in a 1×30 cm. tube as described by Palmer (14). The various acid fractions were collected in test tubes on a fraction collector, evaporated to dryness and titrated with standard base. Tentative identification of the various peaks was made by comparison to the curves obtained by following the above described procedure with a mixture of authentic samples of succinic, malic, citric, and aconitic acids (18, Fig. 1).

The tentative identifications were further substantiated by paper chromatography. Aliquots of solutions from the acid peaks were applied to 25×40 cm. Whatman No. 1 filter paper sheets and run with known acids in solvents A, D, E, and F of Stark, Goodban, and Owens (19), the solvents described by Resnik, Lee, and Powell (16) and by Palmer (14). Co-chromatography with authentic acids was considered tentative identification. Further evidence on ortho-phosphate identification was obtained by the phosphomolybdate reaction (4, 7).

Since malonic and citric acids were eluted simultaneously from the anion exchange column, they had to be separated by chromatography on a silica gel column (5). The acids separated by such chromatography were recrystallized from ether and their identities proved by infrared spectrophotometry, carbon and hydrogen analyses and melting point determinations. Carbon and hydrogen were determined in duplicate on 2 to 3 mg. samples by the Schwarzkopf Microanalytical Laboratory.[2] Infrared curves were obtained by the KBr pellet technique using 0.6 mg. of material in 200 mg.

[2] Schwarzkopf Microanalytical Laboratory, Woodside 77, N. Y.

KBr. Curves were obtained using a Perkin-Elmer Model 13U Infrared Spectrophotometer.

Changes in the organic acid composition of the uredospores during germination were determined on 10.0 g. aliquots of spores. The spores were dusted on a thin film of distilled water in a Petri dish and incubated in the dark at 20° C. for 48 hours before extracting the spores and germ tubes in 80 per cent ethanol as described above. Insofar as could be determined by periodic microscopical examination there was no appreciable contamination by bacteria or other fungi. In view of this, use of a bacteriostatic agent such as aureomycin as used by Shu, Tanner, and Ledingham (17) was considered undesirable since it might have interfered with normal metabolic processes of the uredospores.

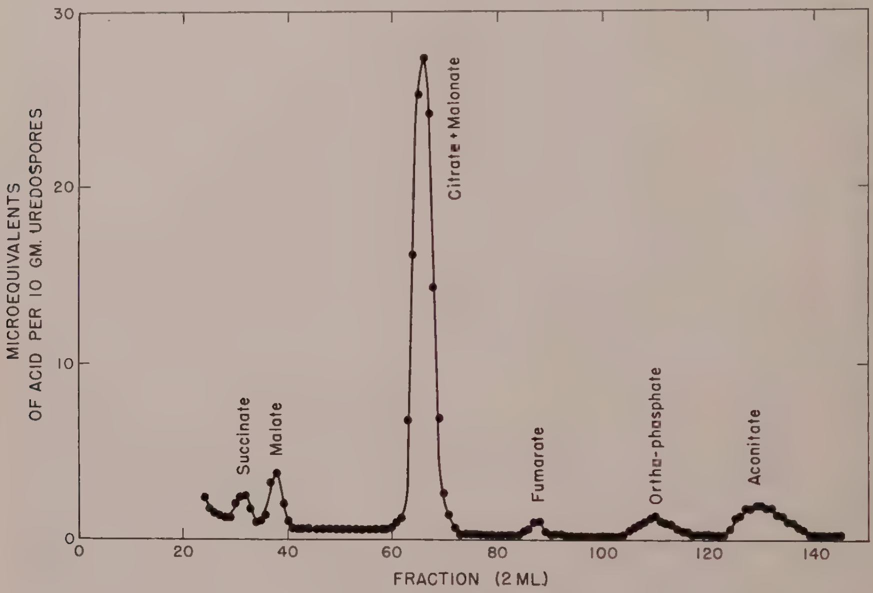

FIGURE 1. Elution curve of acids from resting uredospores of the leaf rust fungus. The spores were extracted with 80 per cent ethanol as described in the text.

RESULTS

WHOLE UREDOSPORES OF THE LEAF RUST FUNGUS

The resting uredospores of the leaf rust fungus were found to contain succinic, malic, citric, malonic, fumaric and aconitic acids. As shown by the titration data in Figure 1, of the fractions eluted from an anion exchange column, the citric-malonic acid fraction predominated. An unknown acid or acids provided a titration peak between malic and citric acids in extracts from some lots of uredospores. This material, which gives a blue color with ninhydrin, is found most commonly in old spores or germinated ones but was not detected in freshly harvested spores.

The aconitate fraction was transferred to paper chromatograms and developed in the solvent mixtures listed in the MATERIALS AND METHODS section. Spots appeared which co-chromatographed with an authentic mixture of *cis-* and *trans*-aconitic acids, thereby demonstrating their presence in the uredospores.

TABLE II

RESULTS OF THE QUALITATIVE ANALYSES OF CRYSTALS OBTAINED FROM UREDOSPORES OF THE LEAF RUST FUNGUS

	Melting points (° C.) (corrected)	
	Malonic acid	Citric acid
Known	135–136	152–153
Unknown	133–134	151–151.5
Mixed	134–134.5	148–152.5

	Carbon-hydrogen analyses (% total)			
	Theory		Found	
	C	H	C	H
Malonic acid	34.6	3.9	34.4	4.0
Citric acid	37.5	4.2	37.7	4.4

The mixture of citric and malonic acids was resolved by chromatography on a silica gel column. The identity of these two materials was substantiated by comparing their melting points, elemental composition and infrared absorption curves to authentic samples as shown in Table II and Figures 2 and 3.

A test made on triplicate 10.0 g. lots of uredospores showed that the citrate-malonate fraction predominated (Table I). Only 25 per cent of this fraction was obtained, however, by ethanolic extraction as shown by the data on ether-extractable acids. When citrate and malonate were separated on a silica gel column they were found to be present in approximately equal quantities. Fumarate and aconitate were present in very limited quantities.

The concentration of the various acids extractable in ethanol changed during germination (Table III). The citrate-malonate fraction eluted from the column increased about 30 per cent but it was established by paper chromatography that malonate had disappeared. It may be deduced, therefore, that citrate had increased from 0.108 milliequivalents of acid to 0.281 milliequivalents (about 2.6-fold). In addition to malonate, aconitate had also been exhausted but there was no detectable change in con-

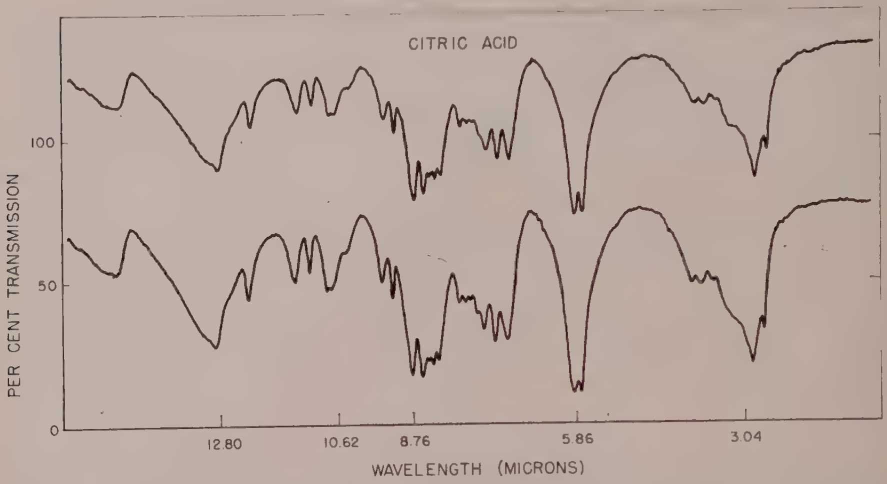

FIGURE 2. Infrared absorption curves of crystals of citric acid from an authentic specimen (lower) and from crystals obtained from leaf rust fungus uredospores (upper). Ordinate refers to the lower curve only.

centration of succinate or malate. It should be noted that the conclusions regarding the change in concentration of citric acid are based entirely upon the ethanol extractable portion of this acid. Although this extraction process is admittedly incomplete, the proportion of acid removed should have been constant because conditions of extraction were rigidly standardized for the resting and germinating spores.

The presence of these six acids suggests that uredospores may have a Krebs cycle or some modification of this. When the various acids (except aconitate) were added to germinating spores only succinate increased the rate of oxygen consumption (Table IV). Because of this, studies were made on the residual acids in uredospores incubated in the presence of succinate. A sample of spores from the stock used in the preceding experiment was shaken for 8 hours in a solution containing 25 ml. of 0.08*M* succinate, 50 ml. of 0.2*M* phosphate buffer at pH 7.0 and 25 ml. water and then analyzed for organic acids after washing it twice in distilled water. The succinate apparently was adsorbed by the spores since the concentration increased 30-fold (Table III). Malate increased threefold and most of the citrate disappeared. Contrary to what happened in germinating spores floated on water, malonate was spared and possibly increased about 40 per cent. The fumarate was not separated from the phosphate fraction so no data were obtained on its concentration. This experiment was not repeated because the supply of uredospores was exhausted.

The effect of various supplements on the respiration of uredospores was determined by Warburg manometry. The addition of 0.08*M* glucose, cit-

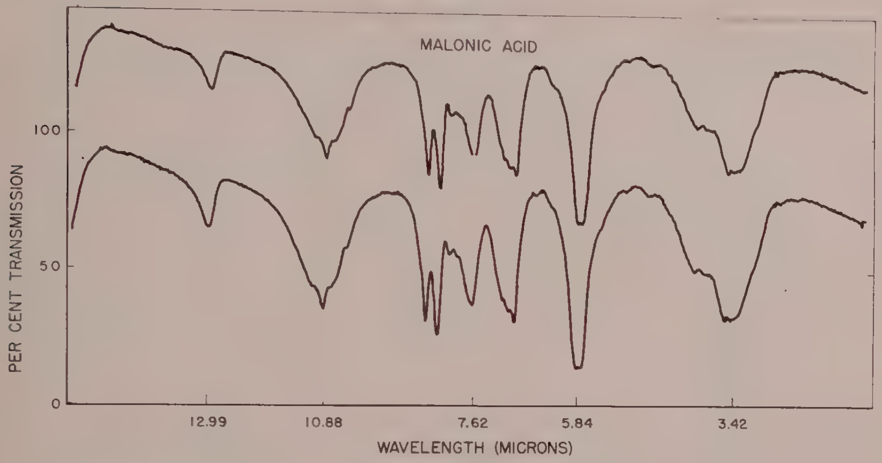

FIGURE 3. Infrared absorption curves of crystals of malonic acid from an authentic specimen (lower) and from crystals obtained from leaf rust fungus uredospores (upper). Ordinate refers to the lower curve only.

rate, malonate, malate, or fumarate did not change appreciably the rate of oxygen uptake in spores (Table IV). However, succinate alone at 0.08*M* increased oxygen consumption about fivefold. This was inhibited when malonate was added with the succinate. Both the endogenous respiration and the respiration of leaf rust uredospores in the presence of succinate were cyanide-sensitive.

The endogenous respiratory quotient (R. Q.), the ratio of carbon dioxide released to oxygen consumed, was 0.42 ± 0.02. It was 0.20 ± 0.02 when spores were incubated with succinate at either 0.08*M* or 0.004*M* and 0.56 ± 0.04 for spores incubated with 0.08*M* succinate plus 0.12*M* malonate. The R. Q. was also 0.56 ± 0.02 for spores incubated with 0.004*M* succinate and 0.004*M* malonate. R. Q. measurements were made at pH 7.0 in 0.2*M*

TABLE III

QUANTITIES OF ORGANIC ACIDS OBTAINED FROM WHOLE UREDOSPORES OF THE LEAF RUST FUNGUS BY EXTRACTION WITH 80% ETHANOL

Acid	Milliequivalents of acid per 10.0 g. spores		
	Resting spores	Germinated spores	Resting spores incubated with succinate*
Succinic	0.017	0.015	0.510
Malic	0.020	0.021	0.067
Citric	0.216	0.281	Trace
Malonic	—	0	0.155
Fumaric	0.003	0.005	—
Aconitic	0.010	0	—

* One analysis.

TABLE IV

EFFECT OF VARIOUS SUBSTRATES ON THE RATE OF OXYGEN UPTAKE OF WHOLE UREDOSPORES. RATIO $\frac{\mu\text{L. } O_2 \text{ UPTAKE PER HOUR IN SUBSTRATE}}{\mu\text{L. } O_2 \text{ UPTAKE PER HOUR IN WATER}}$ FOR 35 MG. WHOLE SPORES

Substrate (0.08M)	Leaf rust fungus	Stem rust fungus
Glucose	1.0	0.9
Citrate	1.0	0.8
Malonate	1.1	0.8
Succinate	5.4	1.9
Malonate+succinate	1.2	1.0
Malate	0.9	1.0
Fumarate	0.9	—
Cyanide	0.2	—
Cyanide+succinate	0.3	—

phosphate buffer on at least two populations of spores replicated four times.

The effect of pH on the rate of oxygen uptake of whole spores in phosphate buffer is depicted in Figure 4. The optimum pH for the succinate-induced oxygen uptake was about pH 6.5, while that for the endogenous respiration of whole spores was pH 6.0. Changing the anion from phosphate to phthalate shifted the optimum pH of the endogenous respiration (Fig. 5).

Response of whole spores to exogenous succinate made it evident that a succinate oxidizing system was present in the spores. If this succinate oxidizing system were succinoxidase, succinate-stimulated oxygen uptake should be inhibited by malonate and cyanide. Inhibition of oxygen uptake by malonate and cyanide is made evident by the data in Table IV. A double reciprocal plot (Fig. 6), made by following the method of Lineweaver and Burk (12), indicated that the inhibition by malonate was competitive.

WHOLE UREDOSPORES OF THE STEM RUST FUNGUS

It was desired to compare the biochemistry of the uredospore of the leaf rust fungus with that of the uredospore of the stem rust fungus, since a limited collection of stem rust uredospores was available.

Ethanol extracts of stem rust uredospores were made, as outlined above, and elution curves constructed. Comparison of an elution curve of a uredospore extract (Fig. 7) to one of an authentic mixture of acids (18, Fig. 1) was followed by paper chromatography of the acid peaks. The six solvents described above were used for the development of the paper chromatograms. The organic acids in the extract were tentatively identified as succinic, malic, citric, malonic, fumaric and aconitic acids.

Data on oxygen consumption by uredospores of the stem rust fungus with various exogenous substrates is given in Table IV. Succinate nearly

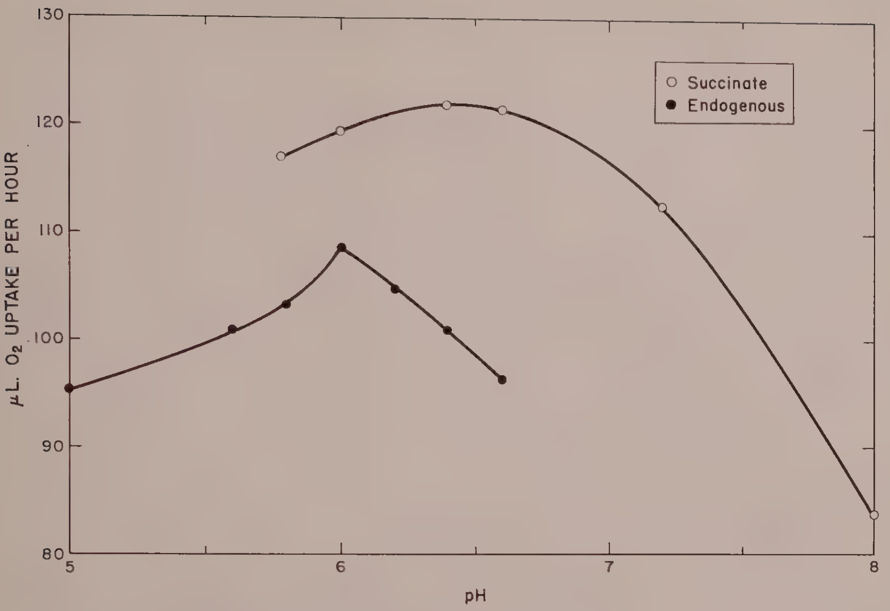

FIGURE 4. The effect of pH on oxygen uptake of whole uredospores of the leaf rust fungus. Measurements made in the Warburg respirometer at 30° C. in phosphate buffer, 0.08*M* succinate.

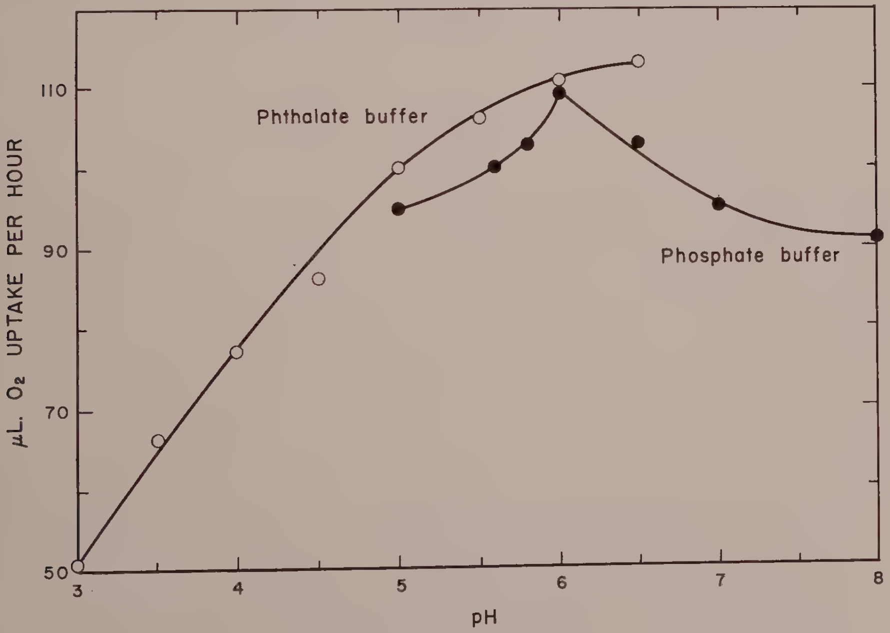

FIGURE 5. The optimum pH of oxygen uptake of whole uredospores of the leaf rust fungus in phthalate and phosphate buffers. Measurements were made in the Warburg respirometer at 30° C.

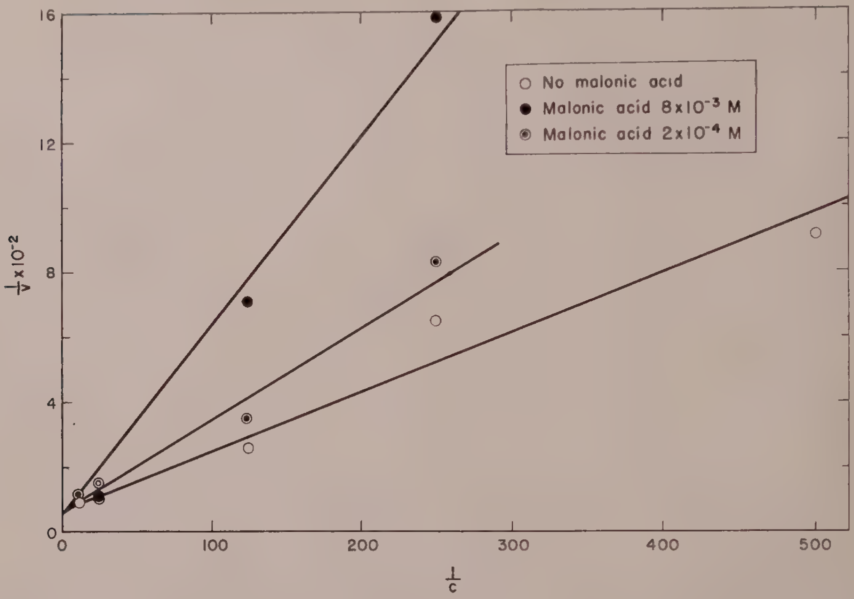

FIGURE 6. A double reciprocal plot of the effect of succinate (c=concentration in moles per liter) with and without malonate on the oxygen uptake by uredospores of the leaf rust fungus in phosphate buffer at pH 7.0. v=μl. O_2 uptake per hour.

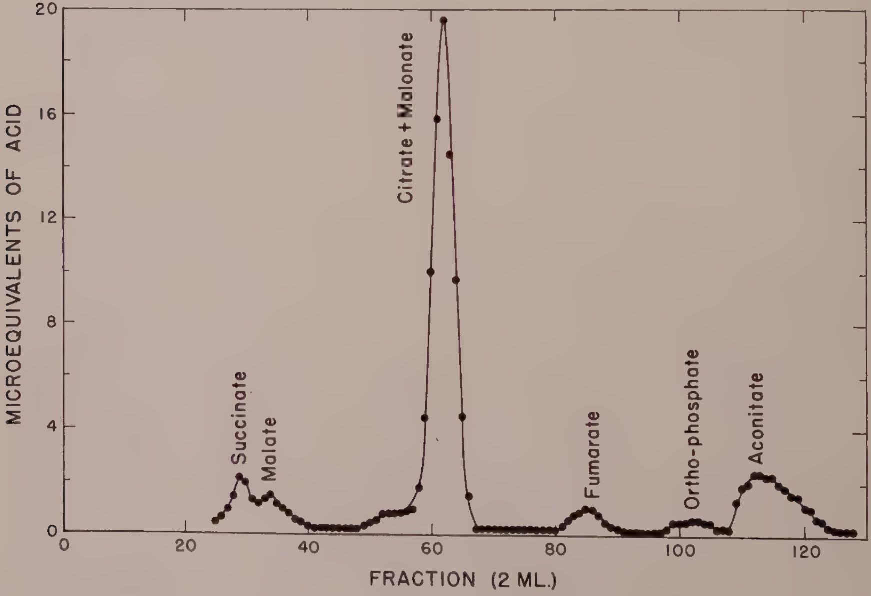

FIGURE 7. Elution curve of acids from resting uredospores of the stem rust fungus. The spores were extracted with 80 per cent ethanol as described in the text.

doubled the rate of oxygen uptake while other substrates did not greatly affect the rate. The data in Table IV also demonstrate that this succinate-induced oxygen uptake was malonate-sensitive.

DISCUSSION

The finding of aconitic, citric, fumaric, malic and succinic acids in uredospores of the two rust fungi suggests that they may have the Krebs citric acid cycle of respiration. The only missing member of the nonketo acids was isocitrate and its absence would not invalidate the hypothesis since it may not accumulate in sufficient quantities to be detected. No tests were made for the keto acids so further work would be required to determine whether they are present. It should be pointed out that demonstration of the intermediates of a metabolic pathway does not in itself prove the existence of the pathway.

The presence of malonic acid in the uredospore raises a question as to its origin. It has been identified in tissues of a number of higher plants (3). It was isolated as early as 1925 from alfalfa tissue (20), from wheat leaves in 1931 (13), and runner bean in 1952 (3). Malonate may be derived from oxaloacetate by the mechanism described for pig heart tissues by Vennesland and Evans (22). However, evidence that oxaloacetate is the precursor for malonate in plant tissues or uredospores remains to be obtained.

Leaf rust fungus uredospores metabolized an exogenous supply of succinate. This succinate oxidizing system was competitively inhibited by malonate (Fig. 6), and was cyanide-sensitive (Table IV) so that this system may be succinoxidase.

Malonate did not inhibit the endogenous respiration of the uredospore though cyanide did (Table IV). The optimum pH of the endogenous respiration was different from the optimum pH of the respiration of spores incubated with succinate (Fig. 4), and there thus is raised the question of the quantitative significance of the succinate oxidizing system in endogenous oxygen uptake by these uredospores. If the succinate oxidizing system proves not to be involved in endogenous respiration of the resting uredospore, it may be separated from the endogenous system by some form of barrier. Endogenous malonate could be conceived to serve as such a barrier between the succinate oxidizing system and the endogenous respiratory process in the resting spore, and in this respect it is interesting that malonate is lost from the spore during its germination. The loss of malonate during germination of the spore may allow utilization of exogenous succinate by the germinated spore via this succinate oxidizing system. It was demonstrated that the citric and eventually the malic acid contents of the wheat leaf are increased upon infection by the leaf rust fungus (18). Perhaps there exists an interaction between the systems for organic acid production and utilization of both parasite and host. The general nature of such interaction cannot be stated until more facts are available.

The pH used for the work reported here (7.0) has been found to be too high for malonate penetration into some intact tissues (10, 11). This difficulty may account for the apparent insensitivity of the endogenous respiration to malonate. However, it is well to remember that malonate did inhibit the succinate-induced oxygen uptake in these studies, so malonate must have penetrated into the spore unless the succinate oxidizing system was on the spore surface. Malonate-insensitivity may, therefore, prove to be a characteristic of the endogenous respiratory process. The observed response of the endogenous respiration to cyanide would imply that at least one metal-containing enzyme was important in the functioning of this process. This is in line with the observations of Shu, Tanner, and Ledingham (17), who observed that uredospores of the stem rust fungus contained materials which gave a positive test with the Nadi reagent.

The respiratory quotient of the uredospores was lowered from 0.42 to 0.20 by incubation with succinate. One explanation for this lowered R. Q. could be that succinate was transformed by the spore to some other compound without decarboxylation. Such a product could have been malate or fumarate. Malate increased threefold in resting spores incubated with succinate (Table III), but it is not known if this malate was derived from the exogenous succinate supply.

The uredospores of the stem and leaf rust fungi exhibited some similar biochemical properties. They possessed the same citric acid cycle intermediates (Figs. 1, 7), both possessed the succinate oxidizing systems (Table IV) and did not show a respiratory response to many metabolic intermediates other than succinate (Table IV). The low respiratory quotient of uredospores of the leaf rust fungus (0.42) is different from that found by Shu, Tanner, and Ledingham (17) for the stem rust uredospore. They made their R. Q. measurements at pH 6.5. The significance of this difference is unknown.

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SENESCENCE OF ROSES. I. CHEMICAL CHANGES ASSOCIATED WITH SENESCENCE OF CUT BETTER TIMES ROSES[1]

Leonard H. Weinstein

SUMMARY

Experiments were carried out to study major chemical changes which occurred in cut Better Times roses during aging. The fresh weight of petals increased until the bloom was fully open, after which it decreased. Petal, receptacle, and leaf tissues decreased in dry weight during senescence, presumably due to respiratory loss. Concentration of cyanidin 3,5-diglucoside in petal tissues increased initially and then fell off. Proteolysis went on uniformly in petals, receptacles, and leaves during aging. Free glucose was rapidly depleted. Malic acid accumulated in petal tissues, especially during the later stage of senescence, while it was lost in other organs. In addition, succinic, citric, and two unknown organic acids were detected in rose tissues. With some exceptions, free amino acids and amides accumulated during early stages of senescence, then decreased. Asparagine was the dominant amino compound in petal, receptacle, and stem tissues. Free ammonia accumulated about 20-fold in petal tissues by the end of the experiment. It is suggested that free ammonia contributes to the "bluing" common to aging red roses.

INTRODUCTION

Among commercially important rose varieties, there is considerable variation in the keeping quality or rate of senescence in cut blooms. At the present time there is little published information of a physiological nature on comparative keeping quality of cut roses, nor is much known of the physiology and biochemistry of cut rose senescence.

Siegelman (14) has reported that at 25° C. roses have a high initial rate of respiration which declines after about 40 hours to a steady value for three days, and then declines again. Similar results have been reported by Giles (9) and by Stoltz (18).

One of the common characteristics of senescence in cut red roses is the change in color of the petal anthocyanins from magenta to a purple or blue color. This pigment change is commonly referred to as "bluing." Currey (6), working with the red rose variety George Dickson, reported the cause of bluing to be a lack of tannins in the cell sap of the petals. Twigg (19) has reported little or no change in tannins of Better Times rose petals during aging. He attributes bluing to an increased ratio of anthoxanthin to anthocyanin pigments, to an increased potassium level, and to a higher pH of petal tissues. Anthocyanin concentration was found to decrease with age.

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[1] This investigation was sponsored by a grant from Roses, Incorporated.

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It should be pointed out that Twigg's results were expressed on a unit area of petal basis, which does not account for normal expansion of the individual petals as the rose opens.

This study was undertaken in order to acquire information on senescence and on keeping quality of cut roses.

MATERIALS AND METHODS

Better Times roses used for these experiments were grown by Mr. Paul Schneeberg, Sayville, Long Island, New York. The roses were subjected to the normal procedures of cutting, grading, and cold storage before being shipped for the experiment. One hundred and fifty roses, having stems 12 to 15 inches in length were used. The roses were cut to eleven inches in length and the stems were placed in distilled water. Fifteen roses were harvested immediately and divided into petal, receptacle (ovary, sepals, pistils, stamens), leaf, and stem fractions. The tissues were weighed and then lyophilized. The roses in water were placed in a room maintained at a temperature between 70° and 75° F. and at a relative humidity of 60 to 65 per cent. The room was lighted with incandescent lamps which provided about 10 foot-candles of light to the roses from 7:00 A.M. to 7:00 P.M., after which the lights were turned off until the following morning. When the rose blooms were about one-half open (44 hours after initiation of the experiment), 15 roses were selected for uniformity and harvested as before. Similar harvests were made when the rose blooms were fully open (after 66 hours—center of bloom exposed, petals fully expanded, color good) and when the blooms were senescent (96 hours—same as fully open but inner petals becoming purple to blue in color and petals ready to absciss).

The lyophilized tissues were weighed and then ground in a semi-micro Wiley mill to pass a 60-mesh screen. The samples were then stored over calcium chloride.

Protein nitrogen was determined by extracting small samples of tissue for 16 hours with a refluxing water-alcohol mixture (22), followed by wet-digestion and distillation in a semi-micro Kjeldahl still (10, pp. 805–806). Free ammonia nitrogen was determined on water extracts of tissues by distillation under reduced pressure (20).

Free amino acids and amides were determined by two-dimensional paper chromatography using the method of Porter, Margolis, and Sharp (12). Free amino acids were extracted from tissues with 80 per cent ethanol at 50° to 55° C. for 30 minutes. The mixture was centrifuged and the supernatant decanted. The residue was reextracted and centrifuged a total of five times. The combined supernatants were taken to dryness under a stream of warm air. The residue was taken up with equal amounts of water and chloroform, thoroughly mixed, and centrifuged. The aqueous layer was removed for chromatography. Aliquots of the samples were spotted on

Whatman No. 3 filter paper and were chromatographed in 70 per cent phenol-water (4 parts of 88 per cent phenol: 1 part of water) in the first direction, and in *n*-butanol-acetic acid-water (45:5:14.5) in the second direction.

Nonvolatile organic acids were determined by the method of Palmer (11). Tissues were extracted in 80 per cent ethanol as for amino acids, and the combined supernatants were passed through a 1×15 cm. column of Dowex 1-X10, 200–300 mesh, formate-form, bedded in absolute ethanol (16). Following sorption, the resin column was washed first with a bed volume of 80 per cent ethanol and then with water. Organic acids were removed from the column by gradient elution with $6N$ formic acid. Two milliliter fractions were collected and tubes were dried under a stream of air in a water bath at 40° C. One milliliter of water and sufficient indicator was added to each tube and they were titrated with $0.01\ N$ or $0.10\ N$ NaOH. Malic acid was isolated by following the procedure used by Staples (16). The titrated tubes representing the malate peak were combined and passed through a Dowex 50 (H-form) column to remove sodium ions. The eluate was decolorized with a minimal amount of Norit-A, filtered through Whatman No. 42 paper, and dried under a stream of warm air. The residue was taken up in successive portions of anhydrous ethyl ether and dried on a watch glass. The infrared spectrum was determined on a Perkin-Elmer Model 13U infrared spectrophotometer in a KBr pellet (0.2 per cent unknown in KBr). The sample was co-chromatographed on Whatman No. 1 paper with an authentic sample of l-malic acid in the solvent F mixture of Stark, Goodban, and Owens (17).

Glucose was determined by the paper chromatographic method of Baar (1), with slight modifications. Sugars were extracted from tissues with 80 per cent ethanol as described earlier. Aliquots of extract were spotted on $10\frac{1}{2}'' \times 17''$ sheets of Schleicher and Shuell No. 598 paper, along with appropriate standard solutions at concentrations ranging from 5 to 200 micrograms. One-dimensional ascending chromatography was carried out in butanol-pyridine-water (125:40:125, upper phase).[2] Each sheet was chromatographed three times. Papers were sprayed with aniline phthalate and were developed in an oven at 110° C. for 15 minutes. Developed spots and suitable blank areas were cut from the papers and were eluted with 4 milliliters of glacial acetic acid at room temperature for 24 hours. Samples were read in a Beckman Model DU spectrophotometer at 480 mμ.

Tannins of petal tissues were determined according to the method of Rosenblatt and Peluso (13). Anthocyanin was isolated from 1 per cent methanolic HCl extracts of petal tissues. The pigment was precipitated by addition of 10 volumes of anhydrous ethyl ether (15). The precipitate was taken up in a small volume of 1 per cent methanolic HCl and reprecipitated. This was repeated 10 times. The precipitate was taken up in a small volume of methanol and mixed with a suitable amount of Whatman cellulose powder. The pigment-cellulose powder mixture was then placed at the top of a 1×60 cm. column of cellulose powder previously washed with butanol-acetic acid-water (40:10:50, upper phase). The pigment was eluted as a single component with the same solvent. The eluate was taken to dryness under a stream of warm air. The pigment was taken up in 1 per cent methanolic HCl and precipitated with ethyl ether as before. The precipitate was then collected and dried by lyophilization. A standard curve was prepared by dissolving the anthocyanin in 0.04 per cent ethanolic HCl, making suitable dilutions, and reading in a spectrophotometer at 534 mμ.

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[2] Block, R. J. Personal communication.

Determination of the anthocyanin concentration of petal tissues was made by measuring the absorbance of 0.04 per cent ethanolic HCl extracts. Criteria for identification of the anthocyanin were: Rf in *n*-butanol-acetic acid-water (40:10:50), maximum absorbance, ultraviolet fluorescence, and a series of color tests which included spraying paper chromatograms with 1 per cent $FeCl_3$, and exposure to ammonia vapors. The anthocyanin was hydrolyzed in 5*N* HCl for 30 seconds, cooled, and the anthocyanidin extracted into isoamyl alcohol, leaving the sugar in the acidic layer. Anthocyanidin was identified by Rf in "Forestal" solvent (acetic acid-conc. HCl-water, 30:3:10) (3), maximum absorbance, distribution in organic reagents (7), and color reactions as described above. The sugar moiety from the glycoside was chromatographed as described earlier for glucose.

All chemical determinations were carried out at least in duplicate.

RESULTS AND DISCUSSION

In an experiment of this type, expression of results on a fresh weight or on a dry weight basis does not accurately portray changes which have occurred in the entire rose bloom as a physiological entity. Table I shows, for example, that although changes in fresh weight of receptacle, leaf, and stem tissues are not great over the 96-hour starvation period, there are considerable changes in fresh weight of petal tissues. Expression of results per gram of dry weight is limited by the fact that one gram of dry weight of receptacle or petal tissues may represent combined tissues from four blooms or one bloom, respectively. Therefore, results have been expressed as changes which occur on a per organ basis, and a summation of data from each organ represents the net change per bloom.

Fresh weights of petals increased until the rose was fully open (66 hours) after which there was a loss in fresh weight prior to abscission (Table I). There were no consistent changes in fresh weights of receptacle tissues. Leaves showed an initial decrease in fresh weight at 44 hours, after which there was no further change. Stem tissues increased in fresh weights

TABLE I

AVERAGE FRESH WEIGHTS, DRY WEIGHTS, AND PER CENT DRY WEIGHTS OF TISSUES DURING SENESCENCE

Fraction	No. hours starvation	Fresh wt./flower, g.	Dry wt./flower, g.	% Dry wt.
Petals	0	6.78	1.04	15.3
	44	8.75	1.00	11.4
	66	9.25	0.92	10.0
	96	7.26	0.88	12.1
Receptacles	0	1.49	0.28	19.0
	44	1.48	0.24	16.3
	66	1.58	0.23	14.4
	96	1.35	0.22	16.1
Leaves	0	2.27	0.60	26.4
	44	1.94	0.55	28.4
	66	1.90	0.51	26.5
	96	1.91	0.51	27.0
Stems	0	1.56	0.43	27.6
	44	1.69	0.45	26.7
	66	1.99	0.50	25.0
	96	1.96	0.50	25.6

during the starvation period. Dry weights of petal, receptacle, and leaf tissues decreased during starvation, while that of stems increased (Table I).

The anthocyanin concentration of petal tissues during aging ranged from 10 to 15 per cent of the dry weight. When expressed on the basis of total petals per bloom, the anthocyanin concentration showed an initial increase from 0 to 44 hours, after which there was pigment loss at 66 hours and at 96 hours (Fig. 1). These results suggest that petal tissues either contain an anthocyanase which increases in activity during aging, or that the anthocyanin-synthesizing mechanism is inhibited with age. Only one anthocyanin is present in tissues of Better Times roses and it has been identified as cyanidin 3,5-diglucoside. The pigment has an Rf of 0.16 in butanol-acetic acid-water which corresponds well with results reported by Bate-Smith (2). The pigment turned blue when exposed to ammonia vapor, when sprayed with 1 per cent $FeCl_3$, and when sprayed with Na_2CO_3. The absorption maximum of the pigment in 0.04 per cent ethanolic HCl was 534 mμ. When dissolved in 0.04 per cent ethanolic HCl containing 0.2 per cent $AlCl_3$, the absorption maximum shifted to 572 mμ (Fig. 2). Geissman *et al.* (8) have reported that aluminum salts cause an increase in the absorption maximum of cyanin due to complex formation of aluminum with free 3′,4′-dihydroxy grouping on the phenyl nucleus. The aglycon of the anthocyanin had an Rf in the "Forestal" solvent of 0.54 which corresponds well with that reported for cyanidin by Bate-Smith (3). Co-chromatography of the sugar moiety with glucose, galactose, and mannose indicated that only glucose was present.

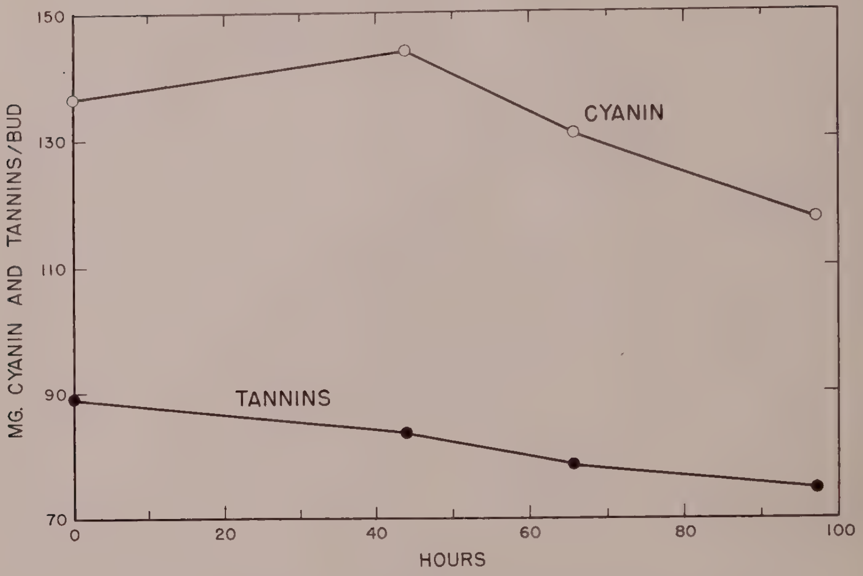

FIGURE 1. Changes in concentration of anthocyanin and tannins of Better Times petals during senescence.

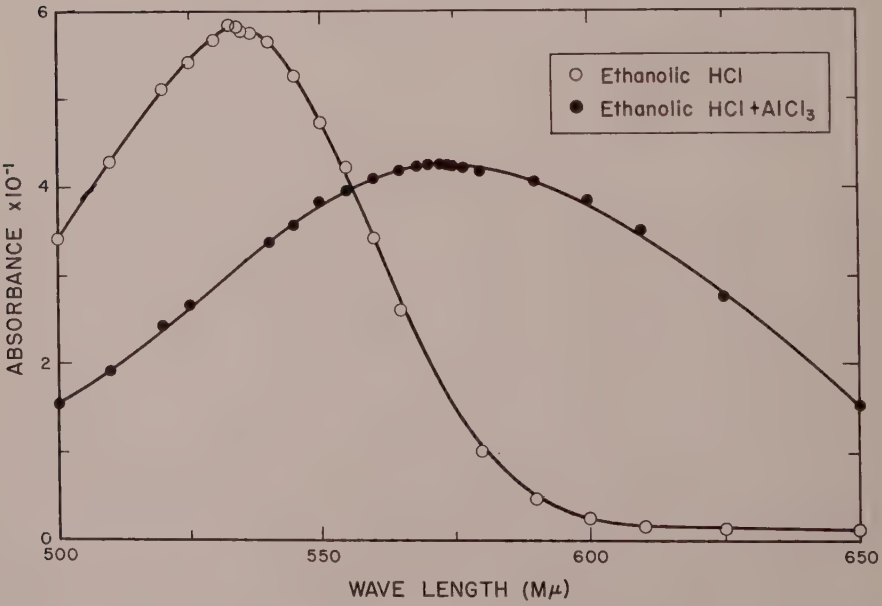

FIGURE 2. Absorption spectrum of Better Times petal anthocyanin in ethanolic HCl with and without aluminum chloride.

In addition to being a component of the anthocyanin, glucose was found to be the major sugar constituent of the petal tissues. Extracts of petal tissue treated with invertase showed little increase in glucose content by paper chromatography. Free reducing substances did not increase significantly. Sucrose is therefore not a major constituent of petal tissues. When paper chromatograms of tissue extracts were treated with aniline phthalate, however, two pink spots and a brown spot were revealed, indicating the presence of pentoses and another hexose. These spots had Rf values corresponding to those of ribose, xylose, and mannose. Results of glucose analyses (Fig. 3) show that during the first 44 hours of starvation petal tissues maintained a high level of glucose, but that it decreased in receptacle and stem tissues.

Since the mechanism for glucose synthesis has in all probability been disrupted by detaching the rose bloom from the plant, and by the subsequent handling and storage treatment, it seems likely that there was a translocation of glucose from receptacle to petal tissues during the first 44 hours. Between 44 and 66 hours of starvation, there was a large decrease in glucose content of petals (76 per cent) and lesser or no decreases in receptacle (38 per cent), leaf (0 per cent), and stem (0 per cent) tissues. By 96 hours, petal and receptacle tissues were nearly depleted of glucose. Leaf and stem tissues also contained a low level, but the proportionate loss was not nearly as great. With consideration of the original level of glucose in each organ and its rate of disappearance during starvation, this hexose may be considered to play a more important role in the metabolism of petal and receptacle tissues than in leaf and stem tissues. If the total glucose content of the entire bloom is considered (Fig. 3), it is found that from 0 to 44 hours there is a loss of glucose of 20 per cent, from 0 to 66 hours of 70 per cent, and from 0 to 96 hours of 86 per cent.

During the initial stages of senescence, while petal and receptacle tissues still contained a relatively high level of glucose, proteolysis went on at a fairly constant rate (Fig. 4). Rate of proteolysis in leaf tissues was also fairly constant but stem tissues showed an erratic pattern. It is obvious from these results that proteolysis proceeds early in the senescence process in cut roses, and that it is independent of the level of a readily available respiratory substrate such as glucose. Wood *et al.* (24) with Kikuyu and Sudan grasses, Yemm (25, 26) with barley, and Viets *et al.* (23) with corn, have shown that leaves exhibited loss of protein almost immediately after detachment. This was also associated with losses of hexoses and sucrose (24). Chibnall (5) has reported that rapid proteolysis takes place in detached leaves of runner bean, but that production of adventitious roots along the petiole by treatment with indoleacetic acid reduces the proteolytic rate. In the present experiment, petal tissues lost almost 40 per cent, receptacle tissues about 31 per cent, leaf tissues about 25 per cent, and

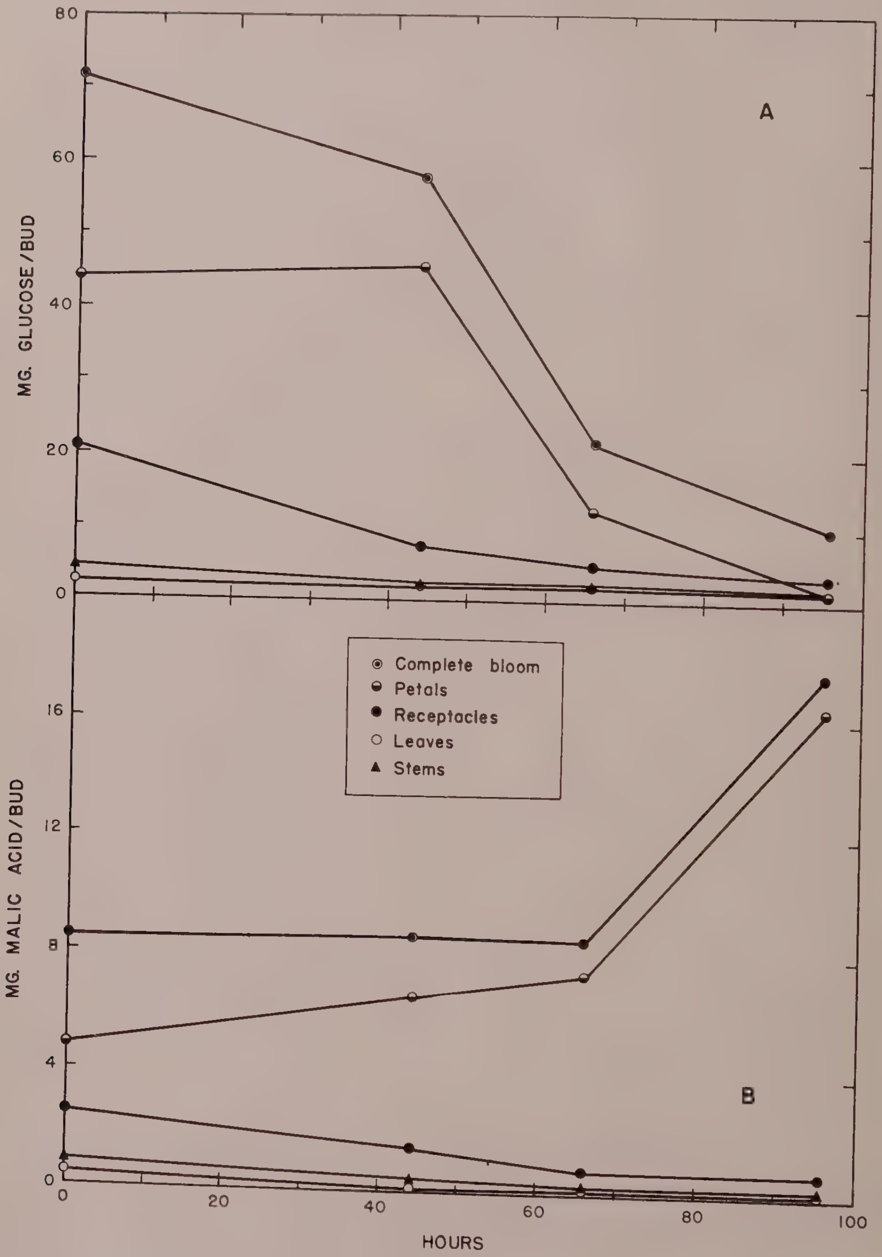

FIGURE 3. Changes in concentration of (A) glucose and (B) malic acid during senescence.

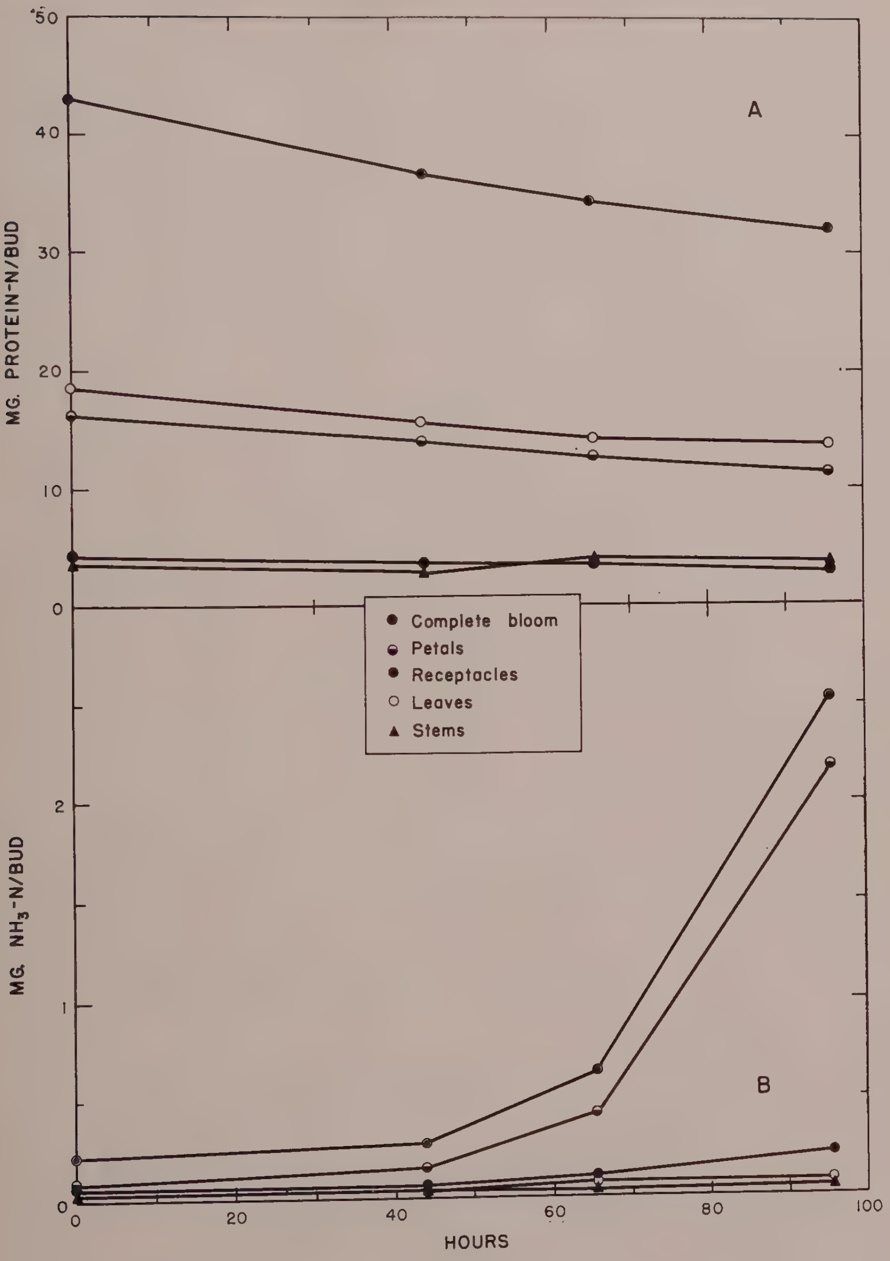

FIGURE 4. Changes in concentration of (A) protein nitrogen and (B) ammonia nitrogen during senescence.

stem tissues about 6 per cent of their original protein nitrogen in 96 hours of starvation. The entire bloom showed a loss in protein nitrogen of about 25 per cent.

Malic acid was found to be the predominant nonketo organic acid of the Better Times roses used in this experiment, making up well over 90 per cent of the total organic acidity. The different organs were found to contain very low levels of both succinic and citric acids. In addition, two unidentified organic acids were detected. One of these acids was eluted from the Dowex 1 column (11) with the fraction corresponding to amino acids, while the other was eluted with the orthophosphoric acid peak. The former material was separated from amino acids by passing the eluate through Dowex 50 resin (H-form). The effluent was taken to dryness under a stream of air, and was then taken up with ethyl ether, leaving behind any inorganic anions which might have been present. The ether was removed by evaporation, and the material was taken up in 80 per cent ethanol and re-chromatographed on a Dowex 1 column. A small peak was eluted in the same position as the original amino acid peak. The eluate was taken to dryness, dissolved in a small amount of water, and chromatographed on Whatman No. 1 filter paper as described earlier. The unknown had an Rf of 0.29 with the Stark, Goodban, Owens solvent F (17).

Changes in succinic, citric, and the two unknown organic acids during senescence were too small to be measured quantitatively. Changes in malic acid are shown in Figure 3. In petal tissues, malate rose gradually during the first 66 hours, after which it rose sharply to a level about 340 per cent greater than its original concentration. In receptacle, leaf, and stem tissues, malate was rapidly depleted during the first 44 hours, and then decreased more gradually during the remainder of the starvation period, with the exception of leaves which increased slightly from 66 to 96 hours. Receptacle, leaf, and stem tissues lost about 71 per cent, 72 per cent, and 70 per cent, respectively, of malic acid during the course of the experiment. Although Figure 3 indicates that petals contain more malate than any other organ, this is due to the greater initial weight of the petals as compared with other organs.

When expressed on a unit of dry weight basis, it was found that malate was present in greatest concentration in receptacles, followed by petals, stems, and leaves. The sum of the malate concentration in each organ (Fig. 3) shows no net change of malate in the total bloom during the first 66 hours of senescence even though there were striking changes in individual organs. At 66 hours, petals of the roses were fully expanded. At 96 hours, the level of malate in petals had increased much more than the corresponding loss from other organs.

It is not known whether the increased level of malate in petals is due primarily to direct translocation from other organs as has been suggested by Bennet-Clark and Woodruff (4) in rhubarb or whether a second distinct mechanism is involved, i.e., a *de novo* synthesis of malate in petals by (a) a CO_2 fixation pathway via pyruvate, (b) transamination between aspartate and a keto acid to form oxalacetate and the corresponding amino acid, and its subsequent conversion to malate by the "malic enzyme," (c) direct formation via fumarate in the Krebs cycle, even though fumarate has not been detected from rose tissues, (d) a combination of synthesis and translocation from other tissues, or (e) some unknown mechanism. In the senescent bloom, malate accumulates to such an extent that losses from other organs do not account for this, and there is a large net increase (106 per cent from 66 to 96 hours). It is likely that this excess malate is being formed, at least in large part, in petal tissues and is not being translocated in its entirety from other organs. Further experiments, which will be reported in a subsequent paper, indicate unequivocally that petal tissues can fix $C^{14}O_2$, which is incorporated rapidly into the malate fraction, and that some rose varieties, at least, contain α-ketoglutarate, pyruvate and possibly oxalacetate. The percentage of total malate accumulated by this pathway, however, has yet to be elucidated. Results do not appear to show that malate synthesis is proportionately linked to glucose degradation, since glucose is depleted most rapidly during the first 66 hours of starvation (Fig. 3), while malate is accumulated most rapidly during the later stages of starvation (66 to 96 hours). Wood *et al.* (24) show an increase in malate in detached Kikuyu and Sudan grass leaves during starvation. In Better Times rose leaves, however, this is not the case. What effect the presence of the petal, receptacle, and stem tissues have on behavior of organic acids in the leaves has not yet been determined.

Figure 5 shows the infrared spectra of l-malate isolated from rose petals and an authentic standard. The unknown had a melting point of 100° to 100.5° C. as compared with 99.5° to 100° C. for l-malate. In addition, malate from petals co-chromatographed with pure l-malate in the solvent system described earlier.

Currey (6) has reported that the tendency for petals of the red-pigmented rose, George Dickson, to turn blue was due to a lack of sufficient tannins in the cell sap of the petals. Twigg (19) has shown that Better Times roses supplied with high nutrient concentrations of potassium blued more extensively and that this was associated with a higher pH value and an increased concentration of an anthoxanthin pigment in the petals, while tannins did not change appreciably. Analyses of tannins of petal tissues in aging roses are shown in Figure 1. Total quantity of tannins in the petals decreased but slightly during senescence. Considering the fact that the ratios of cyanin to tannins at 0, 44, 66 and 96 hours were 1.54, 1.70, 1.67, and 1.57, respectively, remaining relatively constant, and that bluing occurred only at senescence (96 hours), it seems unlikely that the small loss of

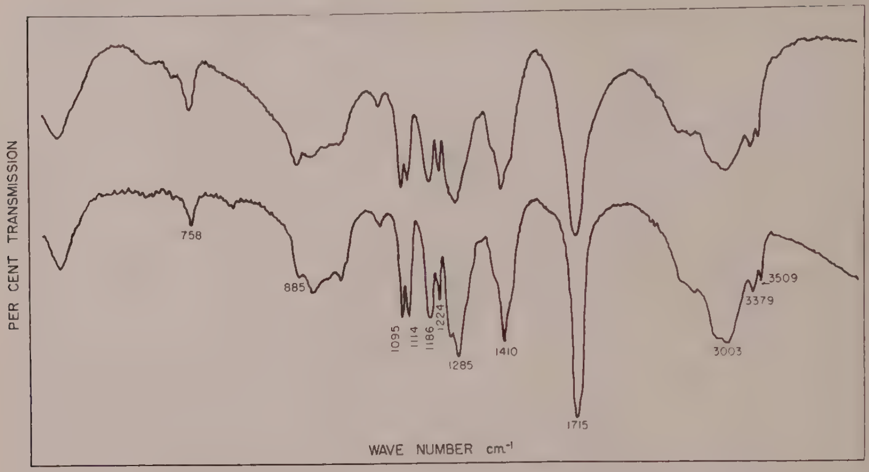

FIGURE 5. Infrared spectra of l-malic acid (upper) and malic acid isolated from petal tissues (lower).

tannins during aging was sufficient to induce bluing, at least in this rose variety. An alternative suggestion would be the influence of free ammonia on ultimate pigment color. Results of ammonia determinations (Fig. 4) showed a 23-fold increase in free ammonia nitrogen during the entire starvation period. From 66 to 96 hours, ammonia nitrogen increased 5-fold. An increase in pH due to the presence of free ammonia should be sufficient to induce bluing. Cyanin is an extremely sensitive indicator of pH, turning violet to blue under slightly alkaline conditions. Since bluing of red roses occurs primarily along the outer edges of the petals, excised blue and non-blue areas from the same petals were analyzed for free ammonia. Blue portions of petals were found to contain 2.3 times as much ammonia as non-blue areas.

In receptacle, leaf, and stem tissues, there was an overall increase in free ammonia nitrogen of 4.3-, 2.0-, and 1.4-fold, respectively, during the course of the experiment.

The increase in free ammonia was coincident with the respiratory loss of certain free amino acids during the later stages of starvation (Table II) and has been reported by a number of investigators (21, 24). The following amino acids and amides have been detected in rose tissues: aspartic acid, glutamic acid, serine, threonine, alanine, valine, the leucines, phenylalanine, γ-aminobutyric acid, tyrosine, glutamine, and asparagine. In addition to these, traces of histidine were detected in petals, arginine in receptacles, an unknown amino acid in leaves, and proline in stems. Asparagine was the dominant amino compound in petal, receptacle, and stem tissues, making up between 65 and 90 per cent of the total amino compounds present. Glutamine was of secondary importance. In leaf tissues,

TABLE II

CHANGES IN FREE AMINO ACIDS DURING SENESCENCE

Amino acid or amide	Mg. amino acid/bloom																			
	Total bloom No. hours				Petals No. hours				Receptacles No. hours				Leaves No. hours				Stems No. hours			
	0	44	66	96	0	44	66	96	0	44	66	96	0	44	66	96	0	44	66	96
Aspartic acid	.29	.26	.18	.17	Tr.*	Tr.	Tr.	.05	.15	.14	.08	.06	.09	.09	.08	.09	.05	.03	.02	.03
Glutamic acid	.84	.83	.59	.57	.41	.48	.31	.29	.20	.09	.06	.06	.18	.19	.14	.14	.05	.06	.08	.09
Serine	.93	1.16	1.37	1.58	.55	.74	1.04	1.21	.07	.05	.06	.09	.24	.29	.19	.19	.07	.08	.09	.09
Threonine	.33	.57	1.10	1.46	.12	.29	.78	1.09	.05	.07	.07	.09	.05	.07	.08	.12	.11	.15	.17	.16
Alanine	.36	.46	.52	.50	.13	.24	.31	.31	.08	.05	.06	.04	.07	.11	.08	.09	.07	.06	.06	.07
Valine	.19	.39	.62	.60	.09	.21	.42	.38	.03	.04	.05	.07	.04	.06	.05	.05	.04	.07	.11	.12
Leucines	.35	.57	.75	.43	.24	.38	.54	.20	Tr.	Tr.	Tr.	Tr.	.06	.08	.11	.09	.06	.11	.10	.14
Phenylalanine	0	.15	1.29	2.73	0	0	1.05	2.17	0	0	Tr.	Tr.	0	.08	.12	.32	0	.07	.12	.24
Tyrosine	.15	1.12	1.87	1.92	0	.54	.99	.67	.03	.11	.12	.16	Tr.	Tr.	Tr.	Tr.	.12	.47	.76	1.09
γ-Aminobutyric acid	.51	.61	.54	.43	.18	.20	.18	.18	.09	.07	.05	.04	.11	.16	.17	.09	.13	.18	.14	.12
Proline	Tr.	Tr.	Tr.	Tr.	0	0	0	0	0	0	0	0	0	0	0	0	Tr.	Tr.	Tr.	Tr.
Arginine	0	.03	.05	.04	0	0	0	0	0	.03	.05	.04	0	0	0	0	0	0	0	0
Histidine	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	0	0	0	0	0	0	0	0	0	0	0	0
Glutamine	2.72	3.13	3.17	2.38	.66	1.00	2.02	1.46	.45	.71	.28	.09	.05	.09	.14	.13	1.56	1.33	.72	.71
Asparagine	46.24	43.72	53.98	52.55	16.55	13.67	21.24	23.94	10.49	8.80	9.09	9.02	Tr.	Tr.	.07	.70	19.20	21.25	23.58	18.90

* Tr. = Trace.

the dominant amino compound was serine during the early period of starvation, and phenylalanine and asparagine during the later stages.

In general, there was a tendency for all amino acids to accumulate during aging as they were released through proteolytic action (Table II). In petal tissues, however, glutamic acid, after a small initial increase at 44 hours, decreased with further aging. Valine, the leucines, tyrosine, alanine, and glutamine each accumulated until the 66-hour harvest, after which they decreased or remained at a constant level. Asparagine decreased at 44 hours, and then accumulated in petal tissues. *Gamma*-Aminobutyric acid remained relatively constant throughout. These results might suggest that after utilization of a preferential respiratory substrate such as glucose, petal tissues first utilized glutamic acid, and later valine, the leucines, tyrosine, alanine, and glutamine. In the case of tyrosine, however, depletion from petals may be accounted for by an increase in receptacle and stem tissues. The lack of tyrosine and phenylalanine at 0 hours, with their subsequent appearance, indicates that these amino acids are not a part of the free amino pool under normal conditions, but appear only under conditions where proteolysis exceeds protein synthesis.

In receptacle tissues, aspartic acid, glutamic acid, alanine, and γ-aminobutyric acid decreased while serine, threonine, valine, and tyrosine increased with starvation. Arginine increased up to 66 hours, then fell off, and glutamine increased up to 44 hours and then dropped sharply. Asparagine decreased slightly at 44 hours, followed by a slight increase but it never reached its initial level. Phenylalanine did not appear in receptacles until after 66 hours, and was then present only in trace quantities. The leucines were present only in trace amounts throughout.

In leaf tissues, threonine, phenylalanine, and asparagine increased with starvation. Glutamic acid, serine, alanine, and valine reached their peak level at 44 hours, and then decreased in amount or remained constant. The leucines, γ-aminobutyric acid, and glutamine reached a peak at 66 hours, then fell off. Aspartic acid and tyrosine remained constant, the latter being present in trace quantities.

In stems, glutamic acid, serine, valine, the leucines, phenylalanine, and tyrosine accumulated with age. Aspartic acid and glutamine decreased with age. Threonine and asparagine reached their highest level at 66 hours and then fell off. *Gamma*-Aminobutyric acid increased until the 44-hour harvest, and then decreased. Alanine and proline remained constant throughout.

The over-all chemical pattern of starvation in cut roses is complicated by the use of extremely heterogeneous experimental material, viz., tissues which are made up of petals, leaves, stems, reproductive organs, sepals, etc. Individually different chemical processes which undoubtedly occur in these organs, the interchange of materials from one organ to another, interorgan hormonal balance as well as other factors, all tend to give a somewhat obscure picture of the senescence patterns in these tissues.

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FLUORINE IN FOOD WITH SPECIAL REFERENCE TO TEA

P. W. Zimmerman, A. E. Hitchcock, and J. Gwirtsman

SUMMARY

A review of the literature showed that in 1932 commercial tea, *Camellia sinensis* Kuntze (*Thea sinensis* L., *Camellia thea* Link.), was known to contain fluorine (F). Since ornamental camellias (*C. japonica* L.) and tea are members of the Theaceae family, comparisons were made of the F in leaves of the two species. The dry leaves of various domestic brands of tea, composed mostly of young leaves, contained 72 to 115 parts per million (p.p.m.) F and Chinese tea 131 to 178 p.p.m. F on a dry weight basis. One sample of fresh leaves of greenhouse-grown tea plants contained 1,530 p.p.m. F on a dry weight basis and the older leaves of ornamental camellias up to 3,062 p.p.m. F. One sample of young leaves of *C. japonica* contained 67 p.p.m. F. Thus both species of *Camellia* have the capacity to accumulate relatively large amounts of F, especially in the older leaves. The infusion (beverage) from one tea bag in 4.5 fluid oz. of water contained 0.8 to 1.7 p.p.m. F after 3 minutes steeping and 1.0 to 2.0 p.p.m. F after 3 minutes boiling.

There are many natural sources of fluorine in food. The leaves of all species of plants which have been analyzed in this laboratory gave positive tests for F. The actual concentration in the tissues may vary from a few parts to more than 1,000 p.p.m., depending upon the species and age of the leaf. Some species, such as the ornamental camellia, are natural collectors of fluorine. Fruit, seeds, and stems of plants contain comparatively low amounts of fluorine (15).

Commercial tea, *Camellia sinensis* Kuntze (*Thea sinensis* L., *Camellia thea* Link.), and *Camellia japonica* L. belong to the family Theaceae. In view of the known high content of fluorine in leaves of *C. japonica* (13, 15), it was considered of interest to investigate the fluorine content of leaves of the closely-related tea plant, hereafter referred to as *Camellia sinensis*. A review of the literature shows that tea (*Camellia sinensis* Kuntze) was known as early as 1932 (9) to contain fluorine. Several workers have reported results of analyses of various commercial teas (1, 2, 11, 14) and have concluded that tea is definitely a fluorine accumulator (10). It has not been mentioned in the literature by other authors that ornamental varieties of camellia (*Camellia japonica* L.) also accumulate relatively large amounts of F.

The earliest report on the F content of tea (9) showed rather low values (less than 1 p.p.m. F) probably due to the lack of a reliable microanalytical method. After the adaptation of the Willard and Winter (12) technique several articles appeared in the literature (1, p. 58; 4; 5; 10) dealing with F in teas from China, Indochina, India, Ceylon, Russia, and Brazil. They re-

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ported amounts varying from several p.p.m. to several hundred p.p.m. F (1, p. 58). The water-extractable fluoride present in tea varied from 29 to 98 per cent, exceeding 50 per cent for most teas. The more important conclusions were as follows. Old tea leaves accumulated considerably more fluoride than young. The high F content in tea was not due to the F content of the soil but depended primarily on the origin and to a lesser extent on the botanical variety or method of processing.

METHODS AND MATERIALS

The analytical scheme was basically the Willard and Winter (12) method with the incorporation of special equipment and procedures adapted for the determination of fluorine in plant tissue and water (3). The analysis of the tea as well as of the tea extract involved ashing of the sample in the presence of the alkaline fixative (fluorine-free CaO) in an electric muffle furnace at 600° C., fusion of the resulting ash with a five-fold amount of sodium hydroxide at the same temperature, separation of the fluorine from interfering substances by a perchloric acid steam distillation of the caustic melt at $135° \pm 2°$ C. in the presence of silica and silver ions (6), and determination of the fluoride ion by the thorium nitrate tiration in the presence of sodium alizarinsulfonate at $pH = 3.0$ using the photoelectric end-point determination (7). The conversion of the total fluorine into soluble fluoride ion can also be accomplished by a direct fusion of the sample with sodium peroxide in a Parr bomb (8).

Materials for investigation included a number of varieties of camellia leaves, the most common commercial brands of tea used in the United States, two varieties of tea from China, and two varieties of tea from Ceylon and India. The camellia plants were grown at Boyce Thompson Institute, and leaves of different varieties and age were analyzed. In each case the content of a tea bag (approximately 2.5 g.), commonly used in this country by tea consumers, was chosen as a convenient sample for analysis and the same amount was used in each experiment involving steeping or boiling for a given time. The volume of water used for infusions corresponded to the capacity of a commonly used average teacup (4.5 fluid oz., 133 ml.).

EXPERIMENTAL RESULTS AND DISCUSSION

The fluorine content of leaves of several varieties of *Camellia japonica* L. is shown in Table I. It ranges from 67 p.p.m. F for young leaves of *Camellia japonica* L. var. Chandleri Elegans to 3,062 p.p.m. F for old leaves of the same variety. Variations in the fluoride content are probably due to varietal and age differences. Some of these samples were fixed with CaO at the time of collection and others were dried before fixing. Cooperative analyses of two laboratories showed good agreement in results.

TABLE I
FLUORINE CONTENT OF CAMELLIA LEAVES

Variety and species	Date sampled	Treatment	P.p.m. F on dry weight basis
C. japonica L.			
Chandleri Elegans	Oct., 1951	Grown in soil, greenhouse	2065
Chandleri Elegans	Oct., 1951	Grown in gravel and nutrient solution, greenhouse	1630
Chandleri Elegans	Apr., 1952	Grown in soil, greenhouse, leaves washed	3060
Chandleri Elegans	Apr., 1952	Grown in soil, greenhouse, young leaves washed	65
Chandleri Elegans	Apr., 1952	Grown in soil, leaves washed	2900
Chandleri Elegans	Apr., 1952	Grown in soil, leaves wiped	1600
Alba Plena	Oct., 1951	Grown in soil, greenhouse	1320
Alba Plena	Oct., 1951	Grown in gravel and nutrient solution, greenhouse	2020
Alba Plena	Apr., 1952	Grown in soil, greenhouse, leaves washed	1635
Alba Plena	Apr., 1952	Grown in soil, greenhouse, leaves washed	1745
Alba Plena	Apr., 1952	Grown in soil, greenhouse, leaves wiped	1390
Alba Plena	Jan., 1954	Grown in soil	1150
Lee seedling	Oct., 1951	Grown in gravel and nutrient solution, greenhouse	1530
Lady Marion	Oct., 1951	Grown in gravel and nutrient solution, greenhouse	1410
Unknown variety	Feb., 1956	Grown in soil, greenhouse	790
C. sinensis Kuntze	Oct., 1954	Grown in soil, greenhouse	1530

The fluorine content of various brands of tea is shown in Table II. It varies from 72 to 115 p.p.m. F for the domestic brands, the two varieties of Chinese tea containing 131 and 178 p.p.m. F, the tea from Ceylon containing 81 p.p.m. F, and the India tea grown at an altitude of 5,000 feet containing 82 p.p.m. F. In addition, the fluoride content in a water extract was determined after steeping a tea bag for 3 and 15 minutes and also after boiling for 3 and 15 minutes. The content of the tea bags was analyzed after extraction. Some of the results of these analyses are given in Table II. The differences which appear in comparing the values for total fluoride in dry tea and the sum of the fluoride in the extract and residue are probably due to slight variations in the fluoride content of the bags of the same brand. The fluoride content in the extracts after 3 minutes steeping ranged from 41 to 72 per cent; in a similar experiment after 3 minutes boiling the tea infusions contained 60 to 92 per cent extractable fluoride. Use of distilled or tap water had no effect on the recovery of F. The actual concentration of F in the tea beverage, based on the infusion of a tea bag in a 4.5 oz. cup, ranged from 0.8 to 1.7 p.p.m. F for 3 minutes steeping and from 1.0 to 2.0 p.p.m. for 3 minutes boiling (14). The results obtained with commercial tea are in general agreement with those of other workers (1, p. 58; 2; 11). In addition to the dry domestic teas, a beverage prepared from instant tea was found to contain 0.2 p.p.m. F.

While growing under natural conditions, vegetation could accumulate

TABLE II

TOTAL AND EXTRACTABLE FLUORIDE (EXPRESSED AS F) IN VARIOUS BRANDS OF COMMERCIAL TEA

Samples of brands of commercial tea	Total F, p.p.m., in dry tea leaves	After steeping 3 minutes				After boiling 3 minutes			
		F, p.p.m., dry wt.		F, p.p.m., by vol. in beverage	% F extracted	F, p.p.m., dry wt.		F, p.p.m., by vol. in beverage	% F extracted
		Tea infusion*	Residue**			Tea infusion*	Residue**		
1	90	64	25	1.2	71	80	14	1.4	89
2	72	50	17	0.8	69	61	11	1.0	85
3	109	79	24	1.3	72	91	17	1.5	83
4	115	80	26	1.3	70	95	17	1.6	83
5	99	67	34	1.1	68	91	19	1.6	92
6 (Chinese)	178	90	90	1.7	51	106	64	2.0	60
7 (Chinese)	131	54	69	1.0	41	96	50	1.8	73
8 (Ceylon)	81	55	22	1.0	68	67	17	1.3	83
9 (India-Ceylon)	82	56	33	1.1	63	68	16	1.3	83

* Liquid decanted from loose tea or the liquid in which the tea bag was placed.

** Portion remaining in tea bag or residue after decantation when loose tea was steeped or boiled.

fluorine from both soil and air. Except for a few special species, the amount stored in leaf tissue ranged from 5 to 25 p.p.m. However, when fumigated with hydrofluoric acid gas, the amount accumulated by leaves varied with the concentration of fluoride in the air stream. For example, control leaves of tomato (*Lycopersicon esculentum* Mill.) contained 13 p.p.m. of fluorine on a dry weight basis, while leaves of plants fumigated with 26 p.p.b. of HF in air for 24 hrs. had 174 p.p.m., and those fumigated with 80 p.p.b. had 327 p.p.m. of fluorine in the tissue. Similarly, control alfalfa (*Medicago sativa* L.) leaves contained 15 p.p.m., whereas leaves fumigated for 8 days with 1 to 6 p.p.b. of HF contained 512 p.p.m., and in another case the leaves of plants fumigated for 8 days with 1 to 4 p.p.b. contained 703 p.p.m. F in the tissue. Celery (*Apium graveolens* L.) leaflets contained 10 p.p.m. in the control, 224 when fumigated for 6 days with 23 p.p.b. HF, and 905 p.p.m. when fumigated for 6 days with 33 p.p.b. HF. Fruit and stems of vegetation store comparatively small amounts of fluorine (15). The leaves of an oat (*Avena sativa* L.) plot fumigated for 7 days with an average of 7.7 p.p.b. of HF in the air accumulated 505 p.p.m. of F in the tissue as compared to only 1 p.p.m. for the seed. The data herewith show that different age leaves or other parts of plants naturally collect different amounts of fluorine. The variation of fluorine in commercial tea is, in all probability, due to the variation in the age of the tea leaves and also in the percentage of petiole and stem tissue which may be collected when tea is harvested.

It is interesting to note in Table II that when commercial tea is used for a beverage and made according to directions the actual quantity of fluorine in the liquid ranges from 1 to 2 p.p.m. and that this concentration is essentially what is recommended for fluoridation of drinking water.

So far as indicated by tests which were made in this laboratory, commercial tea does not store fluorine compounds in the organic form. In fact, that holds for all species of plants so far examined excepting the African plant, gifblaar (*Dichapetalum cymosum* Hook.) (1, p. 74).

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THE EFFECT OF LIGHT AND OF THE SHOOT APEX ON THE ACTION OF GIBBERELLIC ACID

A. J. Vlitos and W. Meudt

SUMMARY

Gibberellic acid (GA) was found to overcome the inhibitory effects of red, blue, or green light on the elongation of stems of Alaska pea seedlings. The second internode of intact seedlings treated with GA produced a greater total elongation than comparable, decapitated seedlings. The total elongation induced by GA was roughly inversely proportional to the amount of the shoot apex severed from the seedlings; however, the increases in elongation produced by GA in seedlings lacking the terminal 1, 2, and 4 mm. of the shoot apex were greater, on a percentage basis, than in intact seedlings. These results suggest that the elongation response in Alaska pea seedlings is dependent not only upon GA but also upon the combined activity of growth factors produced in the shoot apex.

INTRODUCTION

Applications of gibberellic acid (GA) to many species of plants result in marked increases in the length of stems or leaves (1, 2, 4, 6). Brian *et al.* (3) have suggested that the effect of GA in stimulating elongation of stem internodes is due primarily to increases in cellular extension rather than to increases in cell divisions.

A comparison of the physiological activity of GA and 3-indoleacetic acid (IAA) has indicated that the two compounds are similar in their physiological effects only insofar as they both result in increased extension growth of wheat coleoptiles (3). Brian and Hemming (2) have recently shown, however, that, in the presence of IAA, GA induces more pronounced elongation of pea internode sections. Kato (5) in a comparative study with both of these growth regulators concluded that their mode of action differed in many important respects. He found that the gibberellins, at concentrations ranging from 1000 to 1 p.p.m., were inactive in the *Avena* coleoptile and slit pea assays. Lanolin pastes containing one per cent gibberellin (A and B) did not induce epinasty or roots when applied to petioles of tomato seedlings. The gibberellins did not induce callus formation on cut surfaces of sunflower or tomato. Neither did they inhibit lateral bud formation of bean seedlings; in fact, the elongation of lateral buds was accelerated. Kato concluded that the physiological activity of the gibberellins was different from that of IAA.

One of the most obvious differences between the physiological activity of GA and that of the classical auxins lies in the pronounced elongation

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response elicited in intact higher plants by exogenously applied GA as compared with the slight effect on elongation that one observes with exogenously applied auxin. A less obvious difference between the two compounds is related to the effect of light on the subsequent physiological response elicited by the two types of substances. GA appears, from published accounts (3, 4), to be much more effective in stimulating the elongation of stems of intact, green plants in the light than it is in inducing the elongation of stems of dark-grown plants or parts of such plants. The classical auxins, on the other hand, are more effective in causing extension of cells in the dark. Therefore, most of the highly sensitive bioassays for auxins containing the indole nucleus and other auxins are more effective in complete darkness. The existence of light-mediated mechanisms for the destruction of exogenously applied auxins has, in part, accounted for the selection of assays which require complete absence of light. However, there is no evidence to date that GA is inactivated by light either *in vivo* or *in vitro*.

The present paper is concerned with a study of the ability of GA to induce elongation of pea (*Pisum sativum* L. var. Alaska) seedlings under light of various wavelengths or in complete darkness. A study has also been made of the interactions between GA and growth factors in the shoot apex in pea seedlings. While the present studies were underway, two papers, pertinent to these studies, have appeared. Lockhart (6) has shown that GA overcomes the red-light inhibition of growth in Alaska pea seedlings. Growth is interpreted from Lockhart's papers to mean cell elongation. The experiments described in the present study confirm Lockhart's report and, in addition, lend support to Brian's (2) recent study illustrating potentiation of stem elongation in pea segments floated in buffer solutions containing combinations of GA and IAA.

INFLUENCE OF LIGHT ON THE ACTION OF GIBBERELLIC ACID

Seed of Alaska pea were germinated in 6-inch clay pots containing moist, washed river sand. Germination and emergence took place in a dark room at temperatures ranging from 21° C. to 25° C. When the emerged seedlings were six days old they were thinned to a uniform stand of ten seedlings per pot and were sprayed, until wet thoroughly, with solutions containing 10 p.p.m. GA (obtained from Imperial Chemical Industries, Ltd.), 10 p.p.m. IAA (obtained from Nutritional Biochemicals Corp.), 0.1 per cent, 0.5 per cent or 1.0 per cent sucrose, or with combinations of sucrose plus GA or sucrose plus IAA. Control plants were sprayed with distilled water. All solutions contained approximately 0.01 p.p.m. of Triton X-155 to facilitate wetting and penetration of the plant cuticle. All manipulations were performed in weak light emanating from a 40-watt, ruby red, photographic safety lamp, or, in some experiments, from a 40-watt, green fluorescent lamp which had been wrapped in three layers each of amber and green cellulose acetate sheets, emitting at 546 mμ (7). Both sources of light were found to be without effect on stem elongation during the short periods of exposure (15 to 20 minutes) involved in the preparative routine. Although red light is usually regarded to be photomorphogenically active it was not noted to be so if the seedlings were exposed to this light source for these short periods.

After the sprays were applied, the treated seedlings were placed for 48 hours either in complete darkness or under red, green, and blue fluorescent lamps (two 40-watt tubes of each). Spectral distribution curves for each lamp are reproduced in Figure 1.[1] It is recognized by the authors that these three different qualities of light are not at equal energies and comparisons could be made more precisely if equal energy values for each lamp are used.

Treated and control seedlings were also placed for 48 hours under a 40-watt, ruby red, tungsten filament lamp. After two days measurements were recorded of the total height, in centimeters, of seedlings grown under the various lights. These experiments were repeated at least three times and, in certain cases, as many as ten times. Results of experiments carried out in red light and in darkness are summarized in Table I.

TABLE I

INCREMENTS OF TOTAL HEIGHT FOR TEN SEEDLINGS OF ETIOLATED ALASKA PEAS TREATED WITH GA, SUCROSE, IAA, AND COMBINATIONS OF THESE. GROWN UNDER RED LIGHT OR IN COMPLETE DARKNESS FOR 48 HOURS

Treatment	Increments of total height (cm.) for ten seedlings grown under	
	Red light	Darkness
Controls	50.7	70.1
GA (10 p.p.m.)	96.5	89.1
IAA (10 p.p.m.)	54.9	66.5
Sucrose (0.5%)	58.0	62.6
Sucrose (1.0%)	58.7	67.0
GA (10 p.p.m.)+sucrose (0.5%)	92.9	76.2
GA (10 p.p.m.)+sucrose (1.0%)	80.2	77.7
IAA (10 p.p.m.)+sucrose (0.5%)	44.6	53.3
IAA (10 p.p.m.)+sucrose (0.1%)	41.1	54.1

Difference required for significance: at 5% level = 19.0; at 1% level = 21.9.

The results of these experiments confirm earlier studies (6, 8, 9). The elongation of the second internode of peas is significantly greater in darkness than in red light. GA (10 p.p.m.) overcomes the inhibition of elongation by red light. IAA (10 p.p.m.), singly or in combinations with sucrose, had little effect upon stem elongation either in red light or in complete

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[1] The authors are indebted to Mr. J. C. Hoffman, Illuminating Engineering Section, Westinghouse Electric Corporation, for supplying spectral curves of the lamps used in these studies.

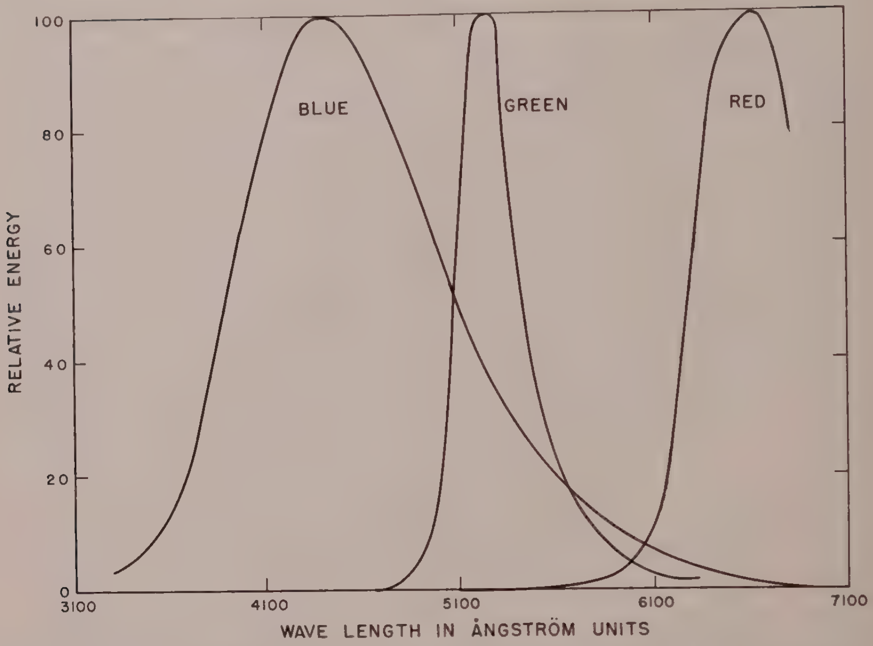

FIGURE 1. Spectral distribution curves for 40-watt blue, green, and red fluorescent lamps.

darkness. However, it caused proliferation of tissues at the nodes of treated seedlings, both in red light and in complete darkness. Combinations of GA and sucrose were effective in red light but not in the dark, but the combination in red light was not more effective than GA used singly. Expansion of primary leaves was generally accelerated by red light, confirming observations of others (6, 8, 9).

The influence of blue, green, and red fluorescent lighting on stem elongation in GA-treated pea seedlings is summarized in Tables II and III.

TABLE II

GROWTH OF SEEDLINGS OF ALASKA PEAS UNDER GREEN LIGHT OR IN DARKNESS FOR 48 HOURS DURING TREATMENT WITH GA AND IAA

Treatment	Increments of total height (cm.) for ten seedlings grown under	
	Green fluorescent	Darkness
Controls	30.3	61.1
GA (10 p.p.m.)	88.3	93.9
IAA (1 p.p.m.)	34.4	80.4
IAA (10 p.p.m.)	28.0	51.3

Difference required for significance: at 5% level = 18.3; at 1% level = 20.6.

The elongation of stems of Alaska pea seedlings was inhibited under green, blue, or red fluorescent lighting. GA at 10 p.p.m. overcame the inhibitory effect of light at these wavelengths. The stimulation of stem elongation by GA in plants grown in complete darkness was much less pronounced than in plants grown under red or blue fluorescent lights. In no instance did GA induce any greater elongation of stems than was found in control seedlings which were sprayed with water and grown in the dark. In contrast, however, the use of IAA at 1 p.p.m. resulted in a significant increase in the elongation of seedlings grown in darkness but was ineffective in stimulating elongation under green fluorescent lighting.

TABLE III

EFFECT OF GIBBERELLIC ACID ON GROWTH OF ALASKA PEA SEEDLINGS EXPOSED TO DIFFERENT LIGHT CONDITIONS FOR 48 HOURS

Treatment	Increments of total height (cm.) for ten seedlings grown under		
	Blue fluorescent	Red fluorescent	Darkness
Controls	40.0	55.6	86.2
GA (10 p.p.m.)	101.7	99.1	111.3

Difference required for significance: at 5% level = 20.0; at 1% level = 22.1.

THE RESPONSES OF DE-ROOTED AND DECAPITATED SEEDLINGS TO GIBBERELLIC ACID

The second internode is the region of maximum elongation in Alaska pea seedlings that are grown in darkness (9) or treated with GA (6). It is of interest to note from published accounts (2, 3), however, that the magnitude of the elongation response elicited by GA is much greater in intact plants than in isolated segments of the same plant, including the second internode of pea. This suggests that either the root system or the apex is in some way implicated in the elongation responses of intact plants that are treated with GA. Experiments were designed to examine this hypothesis.

Alaska pea seedlings were grown in the dark under the environmental conditions described above, but, when they were six days old, ten seedlings for each treatment were either decapitated, by cutting through the first node with a sharp razor, de-rooted, or left intact. The seedlings then were immersed immediately either in aqueous solutions containing 10 p.p.m. GA or in distilled water. One set of seedlings was placed under red fluorescent lights while the other set was kept in complete darkness. After 48 hours their height was measured. The data are summarized in Table IV.

GA overcame the inhibitory effect of red light on the elongation of seedlings possessing intact roots and shoot apices. It had little or no effect on the elongation of decapitated seedlings regardless of whether they were grown in red light or in darkness. GA overcame the inhibitory action of red light on the elongation of de-rooted seedlings with shoot apices. However, de-rooted seedlings, without shoot apices, which are, in effect, second internode segments, did not respond to GA either in red light or in darkness, resembling the response of decapitated seedlings with an intact root system.

The results of these experiments indicate that the shoot apex is necessary for maximum elongation of the second internode of Alaska peas, and that GA will not replace the stimulus for cellular extension that is probably derived from the apex.

A series of experiments was carried out to measure the response to GA of plants which were intact or which were decapitated at varying distances from the primary leaf. Nine sets of six-day-old seedlings were grown and thinned to ten uniform seedlings per pot, as described above, and were either left intact, the primary leaf excised, or 1, 2, 3, 4, 5, 10, and 20 mm. of the apex excised. Intact and decapitated seedlings were then sprayed with 10 p.p.m. GA or with distilled water and maintained under red fluorescent lighting for 48 hours. Height measurements were recorded and the results presented graphically in Figure 2.

TABLE IV

EFFECT OF GIBBERELLIC ACID ON ELONGATION OF ETIOLATED PEA SEEDLINGS WITH DIFFERENT TISSUES REMOVED PRIOR TO TREATMENT IN LIGHT AND DARK

Tissues removed from seedlings	Total increments of growth (in cm.) for ten seedlings held in			
	Red light		Darkness	
	GA (10 p.p.m.)	Water	GA (10 p.p.m.)	Water
Shoot apices	2.4	2.7	4.6	2.0
Roots only	96.6	45.6	107.3	82.6
Roots & apices	5.3	2.1	3.7	2.9
Control	93.2	50.4	106.0	87.3

Difference required for significance: at 5% level = 8.6; at 1% level = 9.7.

The increase in height caused by GA was most pronounced in intact seedlings, but in general, a decrease in total height occurred as the amount of apex removed was increased. Seedlings whose apices were excised at 4 mm. below the primary leaf reversed this general trend, possibly due to the fact that the excision was made either directly above or through a node. In seedlings where 5, 10 and 20 mm. of the apex were excised, growth of controls was inhibited and the response to GA was either slight or nil.

DISCUSSION

These studies suggest that several factors may operate to regulate the elongation of pea seedlings which are treated with GA. The fact that GA overcomes the marked inhibition of elongation by light of various wave-

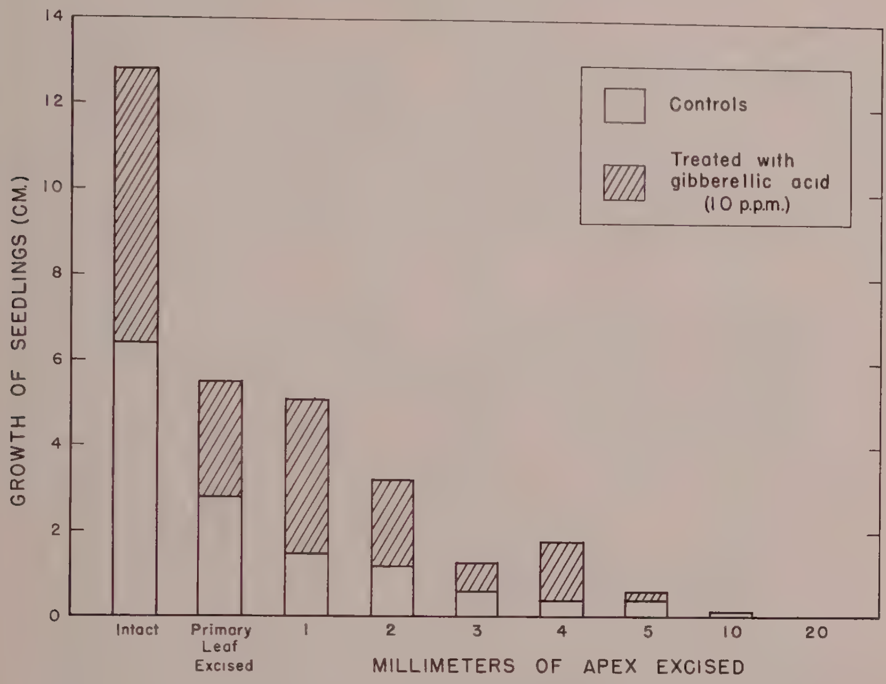

FIGURE 2. Effect of removal of certain tissues on growth of pea seedlings treated with gibberellic acid solutions at 10 p.p.m.

lengths coupled with the much greater total elongation that is produced by GA in plants possessing an intact shoot apex suggests that the activity of GA depends to a great extent on substance(s) which are provided by the shoot apex. These substance(s) may possibly be labile in light.

One of the many possible hypotheses for the phenomena described above is that GA may protect naturally-occurring auxin against light-mediated systems of inactivation resulting ultimately in plants which would contain supra-optimal levels of auxin. However, this is entirely speculative and no data have been presented to substantiate this hypothesis.

Studies have been initiated to determine whether the shoot apex can be replaced by synthetic auxins or by auxin diffused onto agar blocks from the excised apices, and whether GA is dependent upon these factors to produce its maximum etiolation response. Results of these studies will be reported in forthcoming publications.

While the present manuscript was being completed a paper appeared by Lockhart (LOCKHART, JAMES A. Studies on the organ of production of the natural gibberellin factor in higher plants. Plant Physiology **32**: 204–207. 1957) which suggested that applied gibberellin can replace, almost completely, the stem tip in promoting the elongation of the subapical region of the pea stem. Lockhart's data are in agreement with those presented here but the interpretation of the data is not. It is evident from his data that decapitated seedlings treated with GA do not grow to a total height which is equal to or greater than intact, untreated seedlings, unless the decapitated pea seedlings are treated simultaneously with IAA and GA. This may be interpreted to mean that GA, to produce its maximum growth response, is dependent upon the presence of IAA or other growth factors.

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NEMATOCIDAL PROPERTIES OF 3,4-DICHLOROTETRAHYDROTHIOPHENE-1,1-DIOXIDE (PRD)[1]

PAUL H. SCHULDT[2] AND HENRY BLUESTONE[3]

SUMMARY

The *cis* (PRD) and *trans* (ORD) isomers of 3,4-dichlorotetrahydrothiophene-1,1-dioxide as well as the dehydrochlorination product 3-chloro-2,3-dihydrothiophene-1,1-dioxide (FRD) had a high order of nematocidal activity as measured by evaluation against root-knot nematodes (*Meloidogyne* sp.), and *Panagrellus redivivus* (Linn. 1767) Goodey 1945, and by reduction of nematode populations in a field test. These compounds are white crystalline substances with low vapor pressures which probably kill by contact action. PRD and FRD may be slightly more nematocidal than ORD, but all of these compounds kill nematodes rather slowly. PRD and FRD were ineffective against *Panagrellus* at a dosage of 128 p.p.m. when observations were made after two days, but 100 per cent kill was obtained at the end of a week. Similarly, a concentration of 8 μg. of PRD per gram of soil did not reduce populations of root-knot larvae in infested soil within six days, but 75 per cent reduction in population was obtained after two weeks. In greenhouse tests PRD was equal to Vapam (sodium *N*-methyldithiocarbamate) and Nemagon (1,2-dibromo-3-chloropropane) for reducing the number of galls on tomato plants grown in soils infested with root-knot nematodes while in a field test fall applications of PRD were more effective in reducing total nematode population than comparable amounts of Nemagon, Vapam or D-D (mixture of 1,3-dichloropropene and 1,2-dichloropropane). PRD has pre-emergence herbicidal properties and therefore must be used as a pre-planting treatment for many seed-sown crops. However, established tomato, tobacco, and cotton plants tolerated PRD at nematocidal doses.

INTRODUCTION

During investigations of the nematocidal properties of various heterocyclic sulfur compounds it was found that certain halogen substituted derivatives of tetrahydro- and dihydrothiophene-1,1-dioxides were highly active. The compounds of greatest interest were the *cis* and *trans* isomers of 3,4-dichlorotetrahydrothiophene-1,1-dioxide and 3-chloro-2,3-dihydrothiophene-1,1-dioxide, designated hereafter as PRD, ORD and FRD respectively.

PRD is a white crystalline solid with a very low vapor pressure. It is

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[1] These investigations were conducted under the terms of a fellowship established by Diamond Alkali Co., Cleveland, Ohio.

[2] Plant Pathologist, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York. The senior author is indebted to David Lamont for technical assistance.

[3] Research Group Leader, Diamond Alkali Company, Painesville, Ohio.

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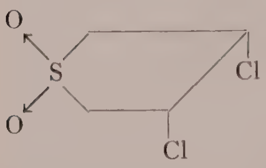
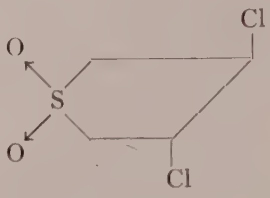
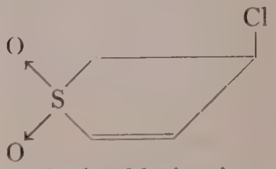
essentially odorless and nonlachrymatory. It does not decompose on dry storage but is unstable to alkalies. Its solubility in water is 0.27 per cent at 25° C. which indicates that it should move downward in soil in response to rainfall (3, 4).



cis (PRD)



trans (ORD)



Dehydrochlorination product (FRD)

This report presents evidence of the ability of these compounds to kill root-knot nematodes (*Meloidogyne* sp.) and *Panagrellus redivivus* by contact action and shows that they are effective in reducing nematode populations in a small scale field test.

MATERIALS AND METHODS

The *cis* (PRD) and *trans* (ORD) isomers of 3,4-dichlorotetrahydrothiophene-1,1-dioxide are prepared by the addition of chlorine to the double bond of butadiene sulfone under varying conditions while 3-chloro-2,3-dihydrothiophene-1,1-dioxide (FRD) is prepared by the dehydrochlorination of either of the former compounds. The preparation of these materials will be described in detail elsewhere. The samples used in these investigations were prepared under the direction of the junior author. Vapam (sodium *N*-methyldithiocarbamate dihydrate), Nemagon (1,2-dibromo-3-chloropropane), D-D (mixture of isomers of 1,3-dichloropropene and 1,2-dichloropropane), VC-13 [*O*-(2,4-dichlorophenyl)-*O*,*O*-diethyl phosphorothionate] and N-521 (tetrahydro-3,5-dimethyl-2*H*-1,3,5-thiadiazine-2-thione) were applied as commercial or experimental formulations.

Nematocidal activity was determined by evaluation against the root-knot nematode (*Meloidogyne* sp.) in soil using tomatoes (*Lycopersicon esculentum* Mill.) variety Bonny Best as the indicator plant, by *in vitro* tests using *Panagrellus redivivus* (Linn. 1767) Goodey 1945, and through observing the reduction in total nematode populations in small field plot tests.

Tomato root-knot test. Composted greenhouse loam soil in ½-gallon glazed crocks was infested with five grams of macerated knotted or galled tomato roots containing egg masses. Chemical treatments were made immediately by mixing solid formulations intimately with the soil, applying liquids as drenches, or injecting fumigants in the centers of the crocks and sealing with water. One week after treatment three tomato seedlings (about 15 to 18 days old) were transplanted into each crock. The degree of knotting or galling of the roots was estimated three weeks after transplanting using a scale of 0 to 4 where 0 equals complete control, 1 equals 1 to 25 per cent of the root tissue galled, 2 equals 26 to 50 per cent, 3 equals 51 to 75 per cent, and 4 equals 76 to 100 per cent (7). The number of pounds per acre was calculated on an area basis and all dosages were expressed on an active ingredient basis.

Panagrellus test. Ten-day-old cultures of *Panagrellus redivivus* grown on cooked oatmeal were exposed to the chemical in small watch glasses (U. S. Bureau of Plant Industry type with dimensions of 27 mm. dia. × 8 mm. deep) within a 9-cm. Petri dish (6, 7). Five watch glasses were placed in each dish; four to measure contact activity at each dosage and one in the center to measure fumigant action. To each 0.4 ml. of solution or suspension of the formulation to be tested, 0.1 ml. of nematode suspension was added making a total of 0.5 ml. per watch glass. The nematode suspension was adjusted so that each 0.1 ml. contained about 25 to 30 nematodes. Water was used in place of the chemical in the watch glasses for fumigant assay. Inactivation counts were made at daily intervals up to 10 days. Because of the extended length of the tests the closed Petri dishes were placed in a saturated atmosphere to prevent drying of the liquid in the watch glasses.

Control of larvae in soil. To determine the concentration of nematocide necessary to kill free living root-knot nematode larvae and the time required to get such action, soil known to be infested primarily with root-knot larvae was mixed carefully, divided into 1500-gram portions, and placed in battery jars (6 × 8 inches). The jars with soil were then stored at about 20° C. for three days in plastic bags to allow eggs to hatch and thereby increase the nematode population. PRD was mixed intimately with the soil in a series of jars at rates of 2, 4, 8, 12, 16, 24, and 32 μg. per g. Control and treated jars were then replaced in the plastic bags and incubated at 22° C. Living nematodes were isolated 1, 3, 5, 6, 7, 8, 9, 10, 13, 14, 19 and 28 days after mixing the chemical with the soil using a modification of the Baermann funnel technique (2, 5). Six tablespoons of soil were placed evenly on a piece of double Kleenex (a paper facial tissue) supported by a circular copper screen cut to fit just inside the top edge of a 5-inch funnel. The outlet of the funnel was closed with a short piece of rubber tubing and a pinch clamp and it was then half filled with water. The soil on the Kleenex was folded into a packet and the soil and screen placed in the top of the funnel. The funnel was filled with water so the soil sample was covered. After two days nematode counts were made by drawing off 25 ml. of water from the funnel and counting the nematodes in three 1-ml. aliquots.

Field evaluation. Field plots, 4 × 4 ft., replicated four times in a randomized block design with a two-foot spacing between all plots, were used to compare the effects of PRD, Vapam, Nemagon and D-D on nematode populations. Applications were made in the latter part of August. All materials were used at rates of 80 and 40 lb. of active ingredient per acre except D-D which was applied at 160 and 80 lb. PRD was incorporated into the top 3 inches of soil, while the other materials were applied by injection into the soil at the depth of 8 inches on 8-inch centers. Two months and eight months after treatment samples were taken with a sampling tube at four soil levels: 0 to 3, 3 to 6, 6 to 9, and 9 to 12 inches. Five borings per plot were made. These were then pooled and the number of nematodes counted as described above.

EXPERIMENTAL RESULTS

LABORATORY AND GREENHOUSE TESTS

Root-knot evaluation. During the last two years an extended series of greenhouse tests has revealed that the *cis* isomer of 3,4-dichlorotetrahydrothiophene-1,1-dioxide (PRD) is very effective for controlling root-knot nematodes. It was comparable to Vapam or Nemagon and considerably more effective than VC-13 on an active ingredient basis (Table I). These data were based on average ratings from several tests and show that PRD under the conditions described, using composted loam soil at a pH of about 6.0, has outstanding nematocidal properties.

TABLE I

SEVERITY OF INFECTION ON TOMATO BY MELOIDOGYNE SP. IN SOIL TREATED WITH FIVE NEMATOCIDES AT DIFFERENT DOSAGES

Dosage lbs./acre active ingredient	Average root-knot index				
	PRD	Vapam	Nemagon	VC-13	D-D
512				1	0
256				4	1
128				4	3
64					4
32	1	1	2		
16	2	2	4		
8	4	4	4		
No. of tests	8	4	3	2	2

Modifications of the root-knot test were used to determine if PRD had the ability to kill root-knot nematodes in galls on living tomato roots and to detect possible systemic activity. To assay for ability to kill root-knot nematodes in galls, single tomato plants were transplanted into soil containing root-knot galls in $\frac{1}{2}$-gallon glazed crocks. After six weeks, when the roots of the tomatoes were heavily galled, PRD was applied to quadruplicate sets of crocks at the rate of 40 and 80 lb. per acre (10 per cent dust formulation) by working the chemical into the top $\frac{1}{2}$ inch of soil.

Six days after treatment and normal greenhouse watering, the plants were removed from the crocks, galled roots were washed clean and 5 grams of minced galls from each of these plants were mixed with sterile soil in new crocks. Young indicator plants were transplanted into this soil immediately, and after the usual three weeks they were removed and the roots were observed for infection. All indicator roots were found to be heavily galled comparable to controls. This would indicate that under the conditions described PRD will not destroy root-knot nematodes once they have entered the root.

To verify the observation that PRD is unable to kill nematodes within galls, and at the same time determine whether side dressings of the chemical are able to reduce infection in established plants, a series of experiments was made in which PRD in a 10 per cent dust formulation was worked into the top ½ inch of soil at various time intervals after transplanting tomatoes into infested soils. Crocks with single indicator plants replicated six times were uniformly watered and roots were examined for galling 53 days after initiation of the test. When 30 lb. per acre was applied 1, 3, 5, 7, 11, and 15 days after transplanting of the indicator plants, average infection ratings of 5, 7, 5, 9, 10, and 9 respectively were recorded on what were classed as old roots. Completely new roots which probably had grown from the crown of the plant since treatment were relatively free of galls particularly on early PRD applications. When the 30-lb. rate was applied in three doses of 5, 10, and 15 lb. at 3, 13, and 23 days after transplanting or when these same amounts were applied in reverse order similar results were obtained. The infection on the old roots and gall-free new roots with infested and noninfested controls are shown in Figure 1. These results serve as additional evidence that PRD will not destroy root-knot nematodes once they become established in living, actively growing roots. However, they also show that new root growth can be kept free of infection after treatment with PRD.

To measure possible systemic action of PRD, three-week-old tomato plants growing in 4-inch clay pots were treated at dosages of 30 and 60 lb. per acre by mixing the chemical in the top ½ inch of soil using care to avoid contact with the stem. Two weeks after treatment the plants were removed shaken free of loose soil and replanted in crocks containing root-knot infested soil. After three weeks the plants were removed and the roots were examined for galls. All plants were heavily infected comparable to the controls; thus PRD appeared to have no systemic effect against root-knot nematodes.

In tests comparing the *cis* (PRD) and *trans* (ORD) isomers of 3,4-dichlorotetrahydrothiophene-1,1-dioxide for the control of root-knot nematodes, it was found that PRD was usually slightly more effective than ORD. Moreover, PRD inhibited the growth of the indicator plants to a slightly greater extent. The average fresh weight of the indicator plants in a typical experiment where the crocks were replicated four times with three plants each (Table II) showed that the *cis* isomer was significantly more inhibitory to growth than the *trans* iosmer, particularly at the highest dose. These data also show that the *cis* isomer is a somewhat more efficient nematocide although good control was achieved with both compounds even at the 12 lb. per acre rate. The appearance of the roots of representative plants (Fig. 2) not only shows the nematocidal efficiency of these chemicals but also illustrates the somewhat more vigorous root system of the plants treated with ORD. In connection with this stunting effect it should be remembered that plants were transplanted only one week after treatment of the soil with chemical. There was no observable injury other than a slowing down of growth.

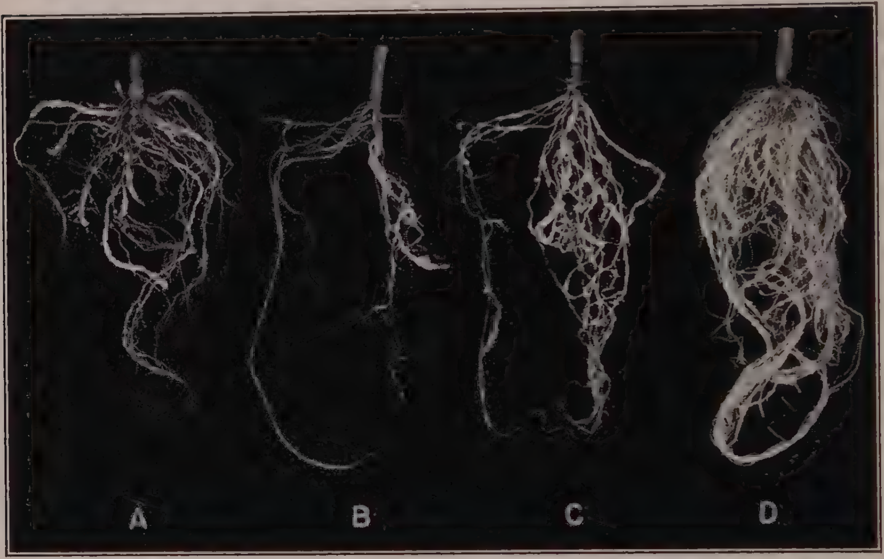


FIGURE 1. Roots of tomato plants showing the effectiveness of PRD when applied as a side dress treatment. (A) Infested control. (B) Treated with 30 lb. per acre of PRD 3 days after transplanting tomato indicator plants. Note gall-free new roots. (C) Treated with 30 lb. applied in 3 doses of 5, 10, and 15 lb. per acre PRD 3, 13, and 23 days after transplanting. (D) Noninfested control.

In other root-knot tests comparing the dehydrochlorination product (FRD) with the *cis* isomer (PRD), no significant difference in nematocidal efficiency was detected. There were differences, however, in phytotoxicity. In laboratory pre-emergence herbicide tests, FRD was found to be more effective than PRD. FRD at 16 lb. per acre gave complete inhibition of broadleaf [turnip (*Brassica rapa* L.), flax (*Linum usitatissimum* L.), and alfalfa (*Medicago sativa* L.)] and grass [millet (*Setaria italica* Beauv.), perennial rye grass (*Lolium perenne* L.), and wheat (*Triticum aestivum* L.)] test species when sprayed on the soil in small 9×9×2 inch aluminum pans 24 hours after planting while 64 lb. per acre were necessary to achieve the same effect with PRD. The weed control properties of these chemicals have also been noted in field evaluations. Since PRD has pre-emergence herbicidal properties it will probably have to be used as a pre-planting treatment for many seed-sown crops. However, after tomato, tobacco and cotton plants have become established, they tend to tolerate PRD when applied to the soil at nematocidal dosages without injury. In general, germinating seed are quite sensitive to PRD but foliage of young seedling plants is very tolerant. PRD was sprayed on the foliage of young tomato plants, four weeks old, and bean, corn and oat plants, two weeks old, at a concentration of 0.64 per cent and no injury was observed.

TABLE II

RELATIVE EFFECTIVENESS OF THE *cis* AND *trans* ISOMERS OF 3,4-DICHLOROTETRAHYDROTHIOPHENE-1,1-DIOXIDE IN PREVENTING INFECTION OF TOMATOES BY ROOT-KNOT NEMATODES

Isomer	Dosage lbs./acre	Chemical μg./sq. cm.	Av. fresh wt./plant, g.	Average rating of infection*
trans (ORD)	48	533	8.0	0
	32	355	8.4	0
	24	266	8.9	1
	16	178	10.8	1
	12	133	13.3	1
cis (PRD)	48	533	3.7	0
	32	355	6.7	0
	24	266	7.9	0
	16	178	7.8	0
	12	133	12.0	1
Infested check		—	7.6	4
Non-infested check		—	20.1	0

* This rating represents an average root-knot index for 12 indicator plants.

Tests against Panagrellus. In early tests it was found that a rather high concentration of PRD was required to kill *Panagrellus redivivus*. After 24 hours' exposure to the chemical the LD50 was found to vary between 500 and 700 p.p.m. while the LD95 was in the neighborhood fo 2000 p.p.m. Since PRD at a concentration of 20 μg. per gram of soil was found to give excellent control of root-knot nematodes it was suspected that factors such as species specificity, activation of the compound in the soil, or insufficient exposure time to the chemical in the *Panagrellus* test might be the cause of this discrepancy.

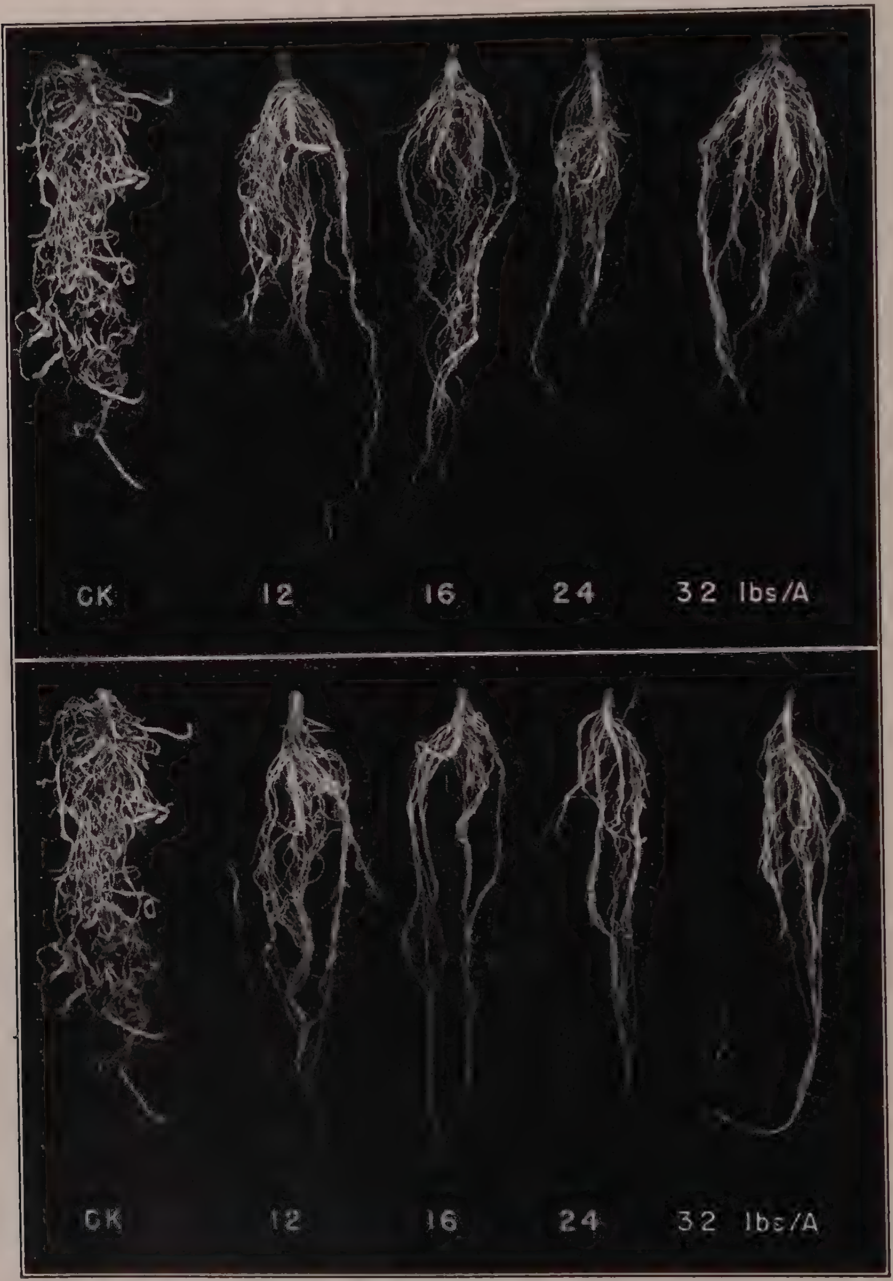


FIGURE 2. Roots of tomato plants illustrating the nematocidal efficiency of the *trans* (top) and *cis* (bottom) isomers of 3,4-dichlorotetrahydrothiophene-1,1-dioxide against root-knot nematode.

In order to evaluate the effects of time of exposure, PRD, its dehydrochlorination product FRD, and experimental compound N-521 (tetrahydro-3,5-dimethyl-2*H*-1,3,5-thiadiazine-2-thione) were tested in concentration series. Observations were made daily on survival of nematodes for a period of eight days. At the end of the first day (Table III) FRD at 1024 p.p.m. had killed 96 per cent of the nematodes while PRD at the same dose killed only 42 per cent. By contrast, N-521 had caused 100 per cent mortality at a dose of 128 p.p.m. while FRD and PRD killed only 15 and 13 per cent respectively at this dosage. However, the nematodes continued to succumb to the cumulative action of FRD and PRD so that by the end of eight days they were substantially equivalent to N-521 in effectiveness at 128 and 64 p.p.m. FRD appeared to inactivate *Panagrellus* more rapidly than PRD, but at the completion of the test virtually complete kill was obtained with both chemicals at the lowest dose.

TABLE III

INACTIVATION RATE AND ULTIMATE NEMATOCIDAL EFFECTIVENESS OF PRD AND THE DEHYDROCHLORINATION PRODUCT (FRD) AGAINST PANAGRELLUS REDIVIVUS

Test material	Concn., p.p.m.	Percentage contact mortality							
		Days after exposure to test chemical							
		1	2	3	4	5	6	7	8
FRD	1024	96	100	100	100	100	100	100	100
	512	81	97	100	100	100	100	100	100
	256	26	70	95	98	99	100	100	100
	128	15	35	82	94	98	100	100	100
	64	14	25	36	56	83	88	95	98
PRD	1024	42	64	73	95	95	99	99	99
	512	16	19	35	83	90	96	98	100
	256	17	17	25	72	85	93	99	100
	128	13	13	19	62	72	86	95	98
	64	12	12	11	29	45	89	87	99
N-521*	128	100	100	100	100	100	100	100	100
	64	93	96	94	93	96	97	98	99
	32	50	74	65	71	66	59	66	61
	16	2	5	5	7	3	7	12	9
Control	—	6	6	7	7	7	7	7	12

* Tetrahydro-3,5-dimethyl-2*H*-1,3,5-thiadiazine-2-thione.

In order to establish which of the two compounds was more active, observations were made on larvae in a geometric concentration series ranging from 4 to 128 p.p.m. and readings made over a 10-day period. Again it was obvious that FRD inactivated the nematodes more rapidly than PRD, but at the end of the test period the two compounds were equivalent. Mortality was higher than in the untreated controls even at the lowest doses, but

TABLE IV

EFFECTIVENESS OF PRD AND ITS DEHYDROCHLORINATION PRODUCT (FRD) AGAINST PANAGRELLUS REDIVIVUS AT LOW CONCENTRATIONS

Test material	Concn., p.p.m.	Percentage mortality								
		Days after exposure to test chemical								
		1	2	4	5	6	7	8	9	10
FRD	128	4	16	58	98	99	100	100	100	100
	64		1	12	62	83	90	93	—	97
	32				34	50	67	76	90	93
	16					31	43	66	86	93
	8						35	43	68	85
	4								51	56
Fumigant control		0	0	0	6	13	18	24	33	39
PRD	128	1	5	20	60	93	99	100	100	100
	64		1	6	39	75	92	98	—	100
	32					41	55	75	71	94
	16					24	28	40	81	87
	8								48	77
	4								43	54
Fumigant control		0	0	0	4	8	13	17	19	26
Control		0	1	2	11	20	23	26	26	30

by this time it was evident that the vitality of the nematodes had been greatly depleted by natural causes (Table IV).

Test against root-knot larvae in soil. In view of the time lag observed in the action of PRD against *Panagrellus*, a test was devised to determine whether a similar effect occurred in the case of root-knot nematodes in soil. Concentrations of 2, 4, 8, 12, 16, 24, and 32 μg. of PRD per gram of infested soil were used and nematode counts made at various time intervals after mixing. The pattern of results (Table V) was the same as those obtained in the studies with *Panagrellus*. Five days were required to obtain 50 per cent kill at 24 μg. per gram of soil. As the concentration of chemical decreased, the time required for 50 per cent kill increased as would be expected. At a concentration of 8 μg. of PRD per gram of soil the population of root-knot larvae was not reduced after six days but after two weeks a 75 per cent reduction was recorded. An interesting point is that even at 4 μg. per gram a 40 per cent reduction in count was recorded after two weeks. At these low concentrations a rather rapid build-up of the nematode population occurred after two weeks which probably meant the PRD had broken down. These results indicate that PRD should give good nematode control in soil at concentrations as low as 16 μg. per gram of soil.

PRELIMINARY FIELD EVALUATION

Additional evidence that PRD is a promising nematocidal chemical

TABLE V

TIME AND CONCENTRATION OF PRD NECESSARY TO KILL ROOT-KNOT NEMATODE LARVAE IN SOIL

Days after mixing PRD with soil	Nematode count from 6 tablespoons of soil expressed as per cent of controls. Concentration of PRD μg./g.							Nematode count of control jar
	2	4	8	12	16	24	32	
1	79	98	108	79	98	116	148	1200
3	77	97	150	124	176	162	118	850
5	91	89	96	96	67	50	46	1150
6	86	89	195	84	57	29	16	1100
7	85	88	61	58	42	11	9	1025
8	71	97	88	57	37	9	6	875
9	62	64	44	49	36	13	5	975
10	59	78	44	45	29	13	9	800
13	53	60	22	31	22	3	4	830
14	71	103	26	31	9	10	2	730
19	118	134	45	39	29	10	6	950
28	124	126	35	42	32	15	7	1010

was obtained from a field experiment designed to measure the relative ability of PRD, Vapam, Nemagon, and D-D to reduce nematode populations in soil when applied in the fall. Two months and eight months after treatment, soil was removed with a sampling tube from each plot at 0 to 3, 3 to 6, 6 to 9, and 9 to 12 inches. Nematode extractions were made and the total count was rounded off to the nearest hundred. The results (Table VI) indicate that under the conditions of this experiment PRD was the most efficient of the nematocides tested on a total count basis. PRD gave better control in the upper levels and the fumigants were more effective at the lower depths. If the fumigants had been injected at a 6-inch depth they might have performed more favorably. Since nematode populations vary greatly within very limited areas, satisfactory sampling is very difficult to achieve. However, that PRD is a potent nematocidal agent that might be useful for pre-planting treatment of infested soils seems conclusive from these data.

DISCUSSION

The *cis* and *trans* isomers of 3,4-dichlorotetrahydrothiophene-1,1-dioxide and the dehydrochlorination product, 3-chloro-2,3-dihydrothiophene-1,1-dioxide, are promising nematocidal agents which have shown very high activity in laboratory evaluations and in preliminary field tests. The *cis* isomer (PRD) and the dehydrochlorination product (FRD) appear to be about equal in nematocidal efficiency although the *cis* isomer is somewhat slower in its action. The similarity of toxic action of these two compounds is not surprising since it is now known that PRD is gradually converted to FRD in the soil. Before this conversion takes place it can be demonstrated

TABLE VI

EFFECT OF SEVERAL NEMATOCIDES ON NEMATODE POPULATION IN A SMALL PLOT FIELD EVALUATION WHEN EXTRACTIONS WERE MADE 2 MONTHS AND 8 MONTHS AFTER TREATMENT

Treatment	Dosage, lbs./acre	Total nematode count (in hundreds) from 6-tablespoon samples taken from four replicated plots at four soil depths				
		0–3 in.	3–6 in.	6–9 in.	9–12 in.	Total
Two months after treatment						
PRD	80	1	9	9	9	28
	40	3	11	15	6	35
Nemagon	80	26	7	5	3	41
	40	32	13	15	9	69
D-D	160	38	8	5	2	53
	80	33	11	9	13	66
Vapam	80	47	15	12	6	80
	40	58	12	8	7	85
Control	—	28	21	16	9	74
Eight months after treatment						
PRD	80	2	1	1	1	5
	40	8	2	4	4	18
Nemagon	80	8	4	1	1	14
	40	12	4	4	2	22
D-D	160	6	2	0	1	9
	80	11	4	2	2	19
Vapam	80	8	5	2	1	16
	40	13	6	3	3	25
Control	—	11	10	5	5	31

that PRD is a less efficient pre-emergence herbicide than FRD. The *trans* isomer (ORD), on the other hand, is slightly less efficient as a nematocide, and is also slightly less phytocidal than PRD. These considerations, together with the fact that a technical product predominating in the *cis* isomer (PRD) is more readily synthesized, suggest that it is the logical candidate for field evaluation as a nematocide.

Since PRD is toxic to seeds and seedlings when used at nematocidal doses it will be best used sometime prior to planting for most seed-sown crops. This is standard practice, since most fumigants used commercially must be applied in advance of planting. However, PRD persists in the soil much longer than these compounds, so it would probably have to be applied in the fall or early spring to avoid injury to the seedlings of some crops.

Early application of PRD might also be advantageous since it would allow a longer time for the downward movement of the chemical in the soil with rainfall, and would permit prolonged contact of the nematodes with the chemical. Following application, PRD would probably decompose slowly to yield nontoxic products that would not injure germinating seed of the crop plant, while in the intervening period it should be able to reduce the stand of weeds.

Concurrent work which will be described at a later date has shown that most crops which have grown past the seedling stage as well as established perennials will tolerate PRD at nematocidal doses of 20 to 50 lb. per acre on an active ingredient basis. This suggests that PRD might find use as a side dressing treatment for ectoparasitic and lesion nematodes.

Since PRD is a solid with a low vapor pressure, has a fairly long residual life in the soil, and kills by contact, this may represent a new approach to the control of nematodes in contrast to the action of fumigants. If so, new methods of application different from those used for fumigants will have to be employed. Recent work on the movement and stability of PRD in soil, employing an analytical procedure described elsewhere (1), indicates that when PRD is applied to the soil surface in late summer or early fall it is evenly distributed by fall rains and winter snows to a depth of 20 inches. Evidence of ability to kill nematodes efficiently when applied in a similar manner is shown clearly in the data presented here. This type of application could eliminate the problems associated with the rush of spring applications as well as the special equipment required to apply fumigants. Most growers have fertilizer spreaders or other equipment which could be used to broadcast PRD over the soil surface in late summer or early fall.

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COLORIMETRIC METHOD FOR THE DETERMINATION OF 3,4-DICHLOROTETRAHYDROTHIOPHENE-1,1-DIOXIDE (PRD)[1]

H. P. BURCHFIELD AND PAUL H. SCHULDT

SUMMARY

A method is described for the colorimetric analysis of the nematocide 3,4-dichlorotetrahydrothiophene-1,1-dioxide (PRD) which depends upon reacting it with 60 per cent pyridine in a boiling water bath and measuring the color which develops on making the solution $M/3$ with respect to sodium hydroxide 18 to 20 hours after cooling. The absorption maximum occurs initially at 439 mμ but shifts to 454 mμ within 20 to 30 minutes after the addition of alkali. Beers' law is followed satisfactorily, and the amount of PRD in solution can be measured to within ±4 per cent. PRD is readily extracted from soil by dichloromethane, and most of the interferences can be removed by adsorption on a mixture of Norit-A and Florisil. Recoveries of 90 to 100 per cent were obtained from soils containing known amounts of PRD. The *cis* and *trans* isomers of PRD as well as its dehydrochlorination product, 3-chloro-2,3-dihydrothiophene-1,1-dioxide, give identical results when absorbances are computed on a molar basis.

INTRODUCTION

The discovery that 3,4-dichlorotetrahydrothiophene-1,1-dioxide (PRD) is effective for the control of root-knot nematodes as well as free living forms (3, 5) prompted investigations on methods for its recovery from soils and determination. Reliable analytical procedures which could be used to detect PRD independently of its biologically inactive breakdown products were particularly desirable since the compound is somewhat phytotoxic, thus making it necessary to study its rate of deterioration in soil, and its movement with rainfall in order to arrive at application methods that would minimize injury to germinating seed.

Since PRD contains chlorine atoms beta to a sulfone group it was believed that these might be sufficiently active to cause the alkylation of pyridine, and thus make possible a colorimetric method based on cleavage of the pyridine ring by sodium hydroxide similar to procedures previously developed for the estimation of some *s*-triazine derivatives (1) and 1-chloro-2,4-dinitrobenzene (4). This was ultimately achieved, although the reaction conditions differ considerably from those used in the analysis of these compounds.

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[1] These investigations were conducted under terms of a fellowship established by Diamond Alkali Co., Cleveland, Ohio.

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MATERIALS AND METHODS

Chemicals. Samples of the *cis* and *trans* isomers of 3,4-dichlorotetrahydrothiophene-1,1-dioxide,3-chloro-2,3-dihydrothiophene-1,1-dioxide,and technical PRD were obtained from Mr. Henry Bluestone of the Diamond Alkali Co. The preparation and properties of these compounds will be described elsewhere. Dichloromethane was obtained from the Eastman Kodak Co., pyridine from the Mallinckrodt Chemical Works, and tetraethylene glycol dimethyl ether from Ansul Chemical Co. Impurities in the glycol ether which inhibited color development were removed by treating it with freshly activated aluminia prior to use. The adsorbant for removing impurities extracted from the soil was a mixture of equal parts of Norit-A (Fisher Scientific Co.) and 60 to 100 mesh Florisil (Floridin Co.). The Florisil was activated by heating at 105° C. for 36 hours before making up the mixture. The reagents used in the analytical determination were *N* sodium hydroxide in water and a 60 per cent by volume solution of pyridine in water.

Extraction from soil. Ten grams of soil (wet or dry basis) were placed in 125 ml. glass-stoppered flasks and shaken for 15 minutes with 20 ml. of dichloromethane on a mechanical shaker. At the end of this time one gram of adsorbant was added and the mixture shaken for an additional five minutes.

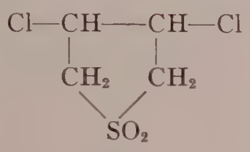
The suspension was then filtered by suction through a medium porosity sintered glass funnel of 30 or 60-ml. capacity into a 125-ml. filter flask. The soil was transferred to the funnel, aggregates broken up with a spatula, and resuspended in 5 ml. of solvent. The filter cake was sucked dry and then resuspended and washed with three 5-ml. portions of solvent. The combined filtrates were transferred to a 100-ml. Kjeldahl flask, 50 μl. of tetraethylene glycol dimethyl ether added, and the solvent evaporated under a current of clean dry air. During evaporation the Kjeldahl flasks were held at 34° C. in a temperature bath.

Colorimetric analysis. Ten ml. of 60 per cent aqueous pyridine were added to each flask, and it was heated for 30 minutes in a boiling water bath. Air condensers consisting of 15-cm. lengths of glass tubing drawn out to capillary tips were used to reduce evaporation losses. After cooling, the solutions were stored for 18 to 20 hours at 22° to 26° C. Two-ml. aliquots were then pipetted into matched 13×100-mm. culture tubes and 1 ml. of *N* sodium hydroxide added to each tube. After 30 minutes the per cent light transmission was determined at 450 mμ with a Bausch & Lomb colorimeter against a blank prepared from a 2-ml. aliquot of the extract containing 1 ml. of distilled water in place of the NaOH. Results were interpolated from reference curves obtained on solutions containing from 1 to 5 μg. of compound per ml. of the final dilution. A set of standards was run with each set of unknowns to compensate for fluctuations caused by temperature differences during the 18 to 20-hour incubation period.

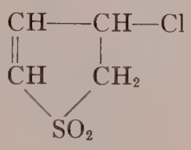
Absorption spectra. Absorption spectra of the reaction products of PRD and 3-chloro-2,3-dihydrothiophene-1,1-dioxide with pyridine and alkali were run in a Model 13 Perkin-Elmer Universal Recording Spectrophotometer one minute and 20 minutes after the addition of alkali. The solutions were incubated for 18 hours at 23° to 26° C. before the addition of alkali. They contained 4 μg. of chemical per ml. of solution at the final dilution.

EXPERIMENTAL RESULTS

Development of method. Technical PRD is a mixture of the *cis* and *trans* isomers of (I) and also contains small amounts of the dehydrochlorination product (II).



(I)



(II)

Since the *cis* isomer of (I) predominates in technical PRD, most of the exploratory work was done with this compound, and the results compared with those obtained on the *trans* isomer of (I), the technical material, and compound (II). The analytical properties of *cis* and *trans* isomers did not differ significantly from one another while the color intensity obtained from (II) was higher on a weight basis but the same on a molecular basis.

On treating (I) or (II) with aqueous pyridine at room temperature and then adding alkali, color was not developed, so evidently the halogens of these compounds are not so labile as those of the dichloro-*s*-triazines (1). However, when pyridine solutions containing 2 to 8 μg. per ml. of (I) or (II) were heated in a boiling water bath for 20 to 30 minutes and then cooled, intense yellow colors with absorption maxima at *ca.* 450 mμ were obtained on making the solutions $M/3$ with respect to NaOH. The color intensity increased with time of heating up to about 20 minutes, and thereafter remained constant. However, the absorbance was found to increase further when the time interval between cooling and the addition of alkali was prolonged. Thus after 18 to 20 hours the absorbance at 450 mμ was more than twice the value obtained when the alkali was added immediately after cooling. Small additional increases in color intensity were obtained on incubating the mixture for an additional 48 hours, but most of the change took place within the first day (Table I). When solutions which had been incubated at room temperature for 18 to 20 hours were re-heated to the temperature of boiling water and alkali added immediately after cooling, the color intensities were reduced to their initial values.

TABLE I

EFFECT OF TIME OF INCUBATION AT ROOM TEMPERATURE ON INTENSITY OF COLOR PRODUCED 30 MINUTES AFTER THE ADDITION OF ALKALI

Time of incubation (hours)	Specific absorbance
0	44
1	62
2	80
3	82
4	88
5	92
20	105
68	108

This indicates that a temperature-dependent equilibrium probably exists between two intermediates, *A* and *B*, such that the formation of *A* is favored at high temperatures and the formation of *B* at low temperatures. Since color intensity and reproducibility of measurement were improved on incubation, all samples were held for 18 to 20 hours at about 23° to 26° C. before adding the alkali.

A third time variable had to be controlled before quantitative results were obtained since it was found that the intensity of color *after* the addition of the alkali increased for the first 20 minutes, and thereafter remained constant for about 20 to 25 minutes before fading began (Fig. 1). This

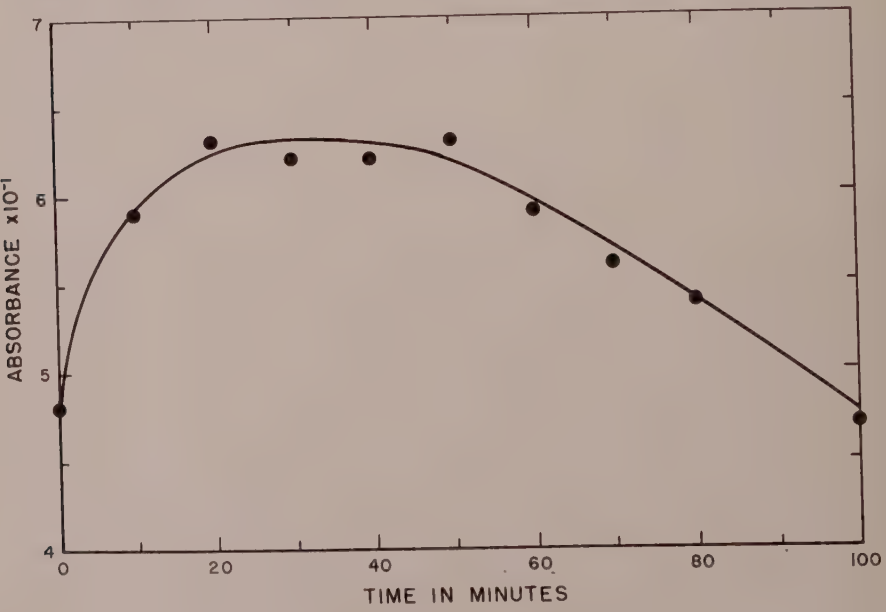


FIGURE 1. Change in absorbance with time after addition of alkali for a solution containing 4 μg. per ml. PRD.

occurred both when the alkali was added immediately after cooling, and when the solution was incubated for 18 hours at room temperature. Measurements made with a Perkin-Elmer Universal Recording Spectrophotometer showed that the change in intensity was accompanied by a shift in the absorption maximum from 439 mμ to 454 mμ. Similar curves were obtained on (II) (Fig. 2) which indicates that it is probably an intermediate in the conversion of PRD to a chromophore. Satisfactory analytical results were obtained at a wave length setting of 450 mμ using a Bausch & Lomb colorimeter 30 minutes after adding alkali.

This method was found to be relatively insensitive to changes in concentration of alkali, but highly sensitive with regard to pyridine concentration. Thus in early work 50 per cent aqueous pyridine was arbitrarily selected as the reagent. It proved to be satisfactory until it became necessary to analyze samples containing 50 to 200 μg. of PRD per ml. These solutions required dilution with fresh pyridine reagent before the addition of alkali in order to reduce concentrations to about 5 μg. per ml. When this was done, recoveries were always low by amounts depending upon the initial concentrations (Table II). On determining color intensity as a function of reagent composition it was found that 60 rather than 50 per cent pyridine was the optimum concentration (Fig. 3). When 60 per cent pyridine was used as the reagent, quantitative recoveries were obtained regardless of the initial concentration of PRD (Table II). In both cases the pyridine was present in large excess so the reasons for this effect are not apparent.

TABLE II

PRD RECOVERY AS A FUNCTION OF THE AMOUNT ORIGINALLY PRESENT IN SOLUTIONS CONTAINING 50 AND 60 PER CENT PYRIDINE

μG. PRD per ml.	Percentage recovered based on standard curve from	
	50% Pyridine	60% Pyridine
50	85	100
100	82	102
150	78	—
200	75	98
400	—	96

When solutions of PRD in 60 per cent pyridine were heated for 20 minutes, incubated at room temperature for 18 hours, and color intensities measured at 450 mμ 30 minutes after making the solutions $M/3$ with respect to NaOH, the average molar absorptivity was found to be 2.2×10^4. The coefficient of variation on a day-to-day basis was 8 per cent. This was reduced to 4 per cent when measurements obtained on the same day were compared. The higher variability of the results obtained on different days was probably caused by differences in room temperature, so it is evident

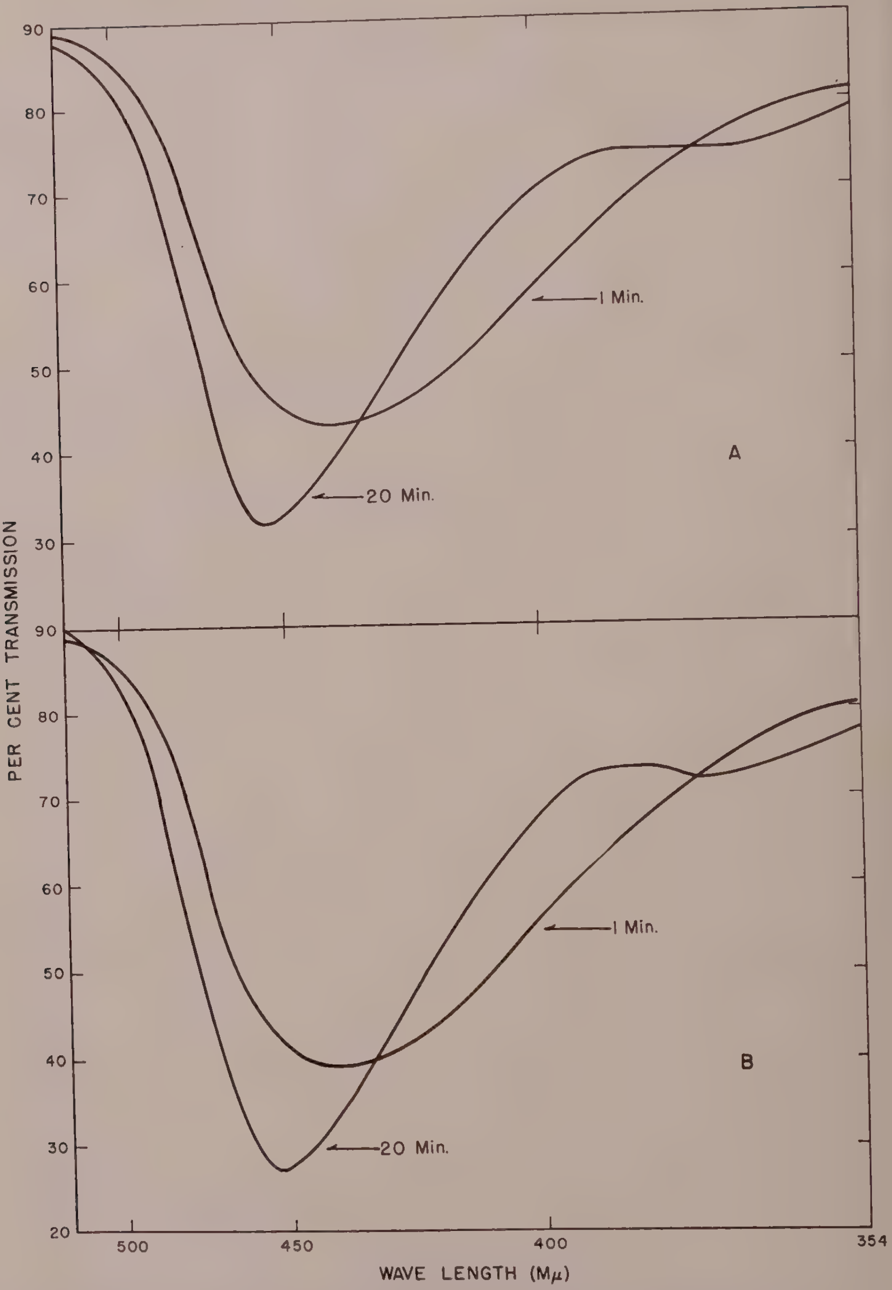


FIGURE 2. (A) Absorption spectra of products derived from PRD at 1 and 20 minutes after addition of alkali at a concentration of 4 μg. per ml. of PRD. (B) Equivalent curves for compounds derived from 3-chloro-2,3-dihydrothiophene-1,1-dioxide.

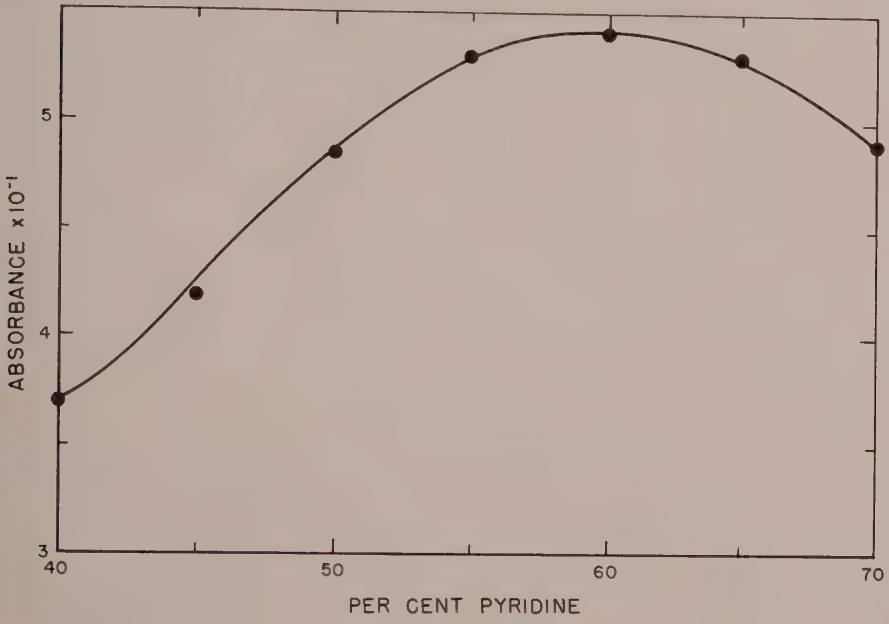


FIGURE 3. Relation between absorbance and concentration of pyridine for a solution containing 4 μg. per ml. of PRD.

that internal standards should be used when equipment is not available for incubating samples at constant temperature.

Extraction from soil. A number of solvents including acetone, carbon tetrachloride, benzene, and dichloromethane were investigated for removing PRD from soil. Acetone was unsatisfactory since it also removed water which subsequently led to losses of PRD during evaporation. Dichloromethane (DCM) proved to be most satisfactory since it has a low boiling point and is a good solvent for PRD. Recoveries of 90 to 100 per cent were obtained when known amounts of PRD were added to composted garden soil (Table III). Recoveries were equally good from both wet and dry soils, while the amount of PRD extracted by carbon tetrachloride was lower for dry soils (Table IV).

TABLE III

RECOVERY OF PRD FROM SOIL BY EXTRACTION WITH DICHLOROMETHANE

Added μg./gram	Recovered μg./gram	Percentage recovered
3	3.0	100
6	6.0	100
9	8.4	93
12	11.6	93
15	13.6	91

TABLE IV

COMPARISON OF DICHLOROMETHANE AND CARBON TETRACHLORIDE FOR EXTRACTING COMPOSTED GARDEN SOIL CONTAINING 50 μG./GRAM OF PRD*

Solvent	Per cent water in soil	Recovered μg./g.	Percentage recovered
CCl_4	4	28	56
	19	39	77
	28	41	82
CH_2Cl_2	4	50	100
	19	57	114
	28	50	100

* Analyses made using 50 per cent pyridine.

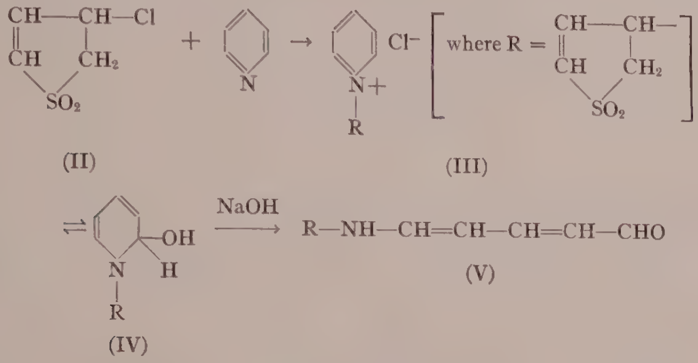
The DCM also extracted colored materials from soils which interfered with the test. Most of these could be removed by adsorption on a mixture of Norit-A and Florisil. It was shown that compounds (I) and (II) were not adsorbed appreciably, and that (I) was not converted to (II) by dehydrohalogenation on treatment with the adsorbant mixture. When this procedure was followed, readings obtained on untreated garden soil used at a rate of one gram of soil per ml. of solvent varied from 90 to 98 per cent transmission against a reagent blank. In order to compensate for this background, absorbances were read against an aliquot of the soil extract which was treated in the same manner as the sample except that one ml. of distilled water was added in place of the *N* NaOH.

Current work indicates that interferences from lipids and pigments extracted from green plants can be eliminated almost entirely by evaporating the DCM and adding petroleum ether. The mixture is then extracted several times with water which removes the PRD while the lipids remain in the petroleum ether phase. The PRD is then extracted from the water phase with DCM and the solvent evaporated.

Losses of PRD during evaporation of DCM under a current of dry air at 34° C. were negligible when a fixative such as tetraethylene glycol dimethyl ether was used, and blowing was interrupted as soon as the solvent odor could no longer be detected. It is important to remove the last traces of DCM since many chlorinated hydrocarbons of this type yield colored products when reacted with pyridine and alkali. However, the solvent is volatile enough so that errors from this source occur very infrequently during routine analyses for PRD.

DISCUSSION

The conditions required to carry out this reaction indicate that the intermediates may be analogous to those proposed for the stepwise reactior of 1-chloro-2,4-dinitrobenzene with pyridine and alkali (2, p. 424; 6) with the exception of the prior conversion of (I) to (II). Thus the ensuing reactions are probably



$$\rightleftharpoons R{-}N{=}CH{-}CH{=}CH{-}CH{=}CHOH \rightarrow OCH{-}CH{=}CH{-}CH{=}CHOH + R{-}NH_2$$

(VI) (VII) (VIII)

where (III) is the quaternary formed by reaction of (II) with pyridine and (IV) is a pseudobase which exists in equilibrium with (III). The experimental results indicate that the equilibrium is probably shifted in the direction of (III) by high temperatures and in the direction of (IV) by low temperatures since color intensity increases on incubation at room temperature (Table I) and decreases to its original value when the solution is reheated. This is based on the assumption that (IV) is the direct precursor of the chromophores (V) and (VI) while (III) must first be converted to (IV). Since (V) and/or (VI) hydrolyze to yield the colorless products (VII) and (VIII), it follows that color intensity will be highest when the rates of formation of the chromophores are highest, and this should occur when (IV) predominates in the equilibrium mixture. Some evidence also exists for the conversion of (V) to (VI) since the absorption maximum produced in the presence of alkali shifts from 439 mμ immediately after addition to 454 mμ 30 minutes later. Following this, the color fades slowly. The probability that glutaconic dialdehyde is one of the final products is indicated by the observation that a rose color is produced when *p*-aminobenzoic acid is added to a solution which has previously been decolorized by heating. This is probably caused by the formation of a monanil or dianil of the aldehyde in the presence of excess amine.

This mechanism cannot be considered to be substantiated by these facts alone, but nevertheless it provides an excellent framework for them and has had considerable practical value for predicting reaction conditions for the analysis of similar compounds containing active halogen atoms.

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BIOCHEMICAL CHANGES INDUCED BY THIOURACIL IN CUCUMBER MOSAIC VIRUS-INFECTED AND NON-INFECTED TOBACCO PLANTS

Clark A. Porter and Leonard H. Weinstein

SUMMARY

Cucumber mosaic virus-infected and noninfected tobacco plants were grown in nutrient solutions containing 0, 3, and 5 p.p.m. of thiouracil, and their leaves were analyzed for certain biochemical constituents. Infected plants exhibited wilting, diffuse mottle, and veinal necrosis in secondarily invaded leaves, and necrotic local lesions on inoculated leaves of the plants grown in thiouracil. The severity of both the local and veinal necroses was increased by thiouracil. Thiouracil toxicity symptoms were manifested as interveinal chlorosis of leaves, as cupped, strapped, and torn leaf laminae, and as discolored and poorly developed secondary roots. Interveinal chlorosis was more severe in noninfected than in infected plants.

Virus infection of tobacco grown without thiouracil induced a stimulation of host metabolism, as compared to noninfected plants, that was reflected by increased dry weight of leaf tissues, and higher levels in leaves of alcochol-soluble amino-, amide-, and ammonia-nitrogen, ribonucleic acid- and deoxyribonucleic acid-phosphorus, malic and citric acids, and a concurrent decrease in the level of nonnucleic acid organic phosphorus.

Both infected and noninfected plants subjected to thiouracil treatment exhibited reduced dry weight of leaf tissues, and lower levels in leaves of total amino-, amide-, and ammonia-nitrogen (soluble and insoluble), organic and inorganic phosphorus, ribonucleic acid- and deoxyribonucleic acid-phosphorus, and malic and citric acids, while there was a higher level of soluble amino acids and amides. Active virus in infected plants subjected to 5 p.p.m. of thiouracil was reduced to nearly one-tenth the concentration present in plants grown without thiouracil.

The data indicate that thiouracil induces a block in the synthesis of proteins; however, a block of the same magnitude did not occur in the synthesis of amino acids and amides. This effect apparently exists because of an aberration in nucleic acid metabolism resulting from the antimetabolic relationship of thiouracil with uracil. It is suggested that the reduction of active virus in thiouracil-treated plants is a consequence of this impaired synthetic mechanism.

INTRODUCTION

Thiouracil has been shown by a number of investigators to be effective in reducing the rate of multiplication of tobacco mosaic virus (TMV) in leaf tissues (1, 8, 9, 12, 18, 20, 22, 23, 24, 29). Its effectiveness toward certain other viruses has also been reported by Bawden and Kassanis (1) who found that it reduced the multiplication in tobacco of all viruses tested, but that it did not affect the development of the Rothamsted tobacco necrosis virus in French bean or of broad bean mottle virus in *Vicia faba*.

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Matthews (20) observed a slight delay in development of systemic symptoms of lucerne mosaic in tobacco treated with thiouracil, but this chemical did not substantially inhibit the systemic development of cucumber mosaic virus in cucumber.

Thiouracil inhibits virus synthesis presumably because of its antagonistic effect toward uracil, since such inhibition can be counteracted by uracil treatment (1, 8, 9). More direct evidence of an antagonistic relationship has been furnished by Jeener and Rosseels (15), Mandel *et al.* (19) and Matthews (21) who have shown the incorporation of thiouracil-S^{35} into TMV particles. The chlorosis and stunting produced by thiouracil when sprayed or watered on intact plants (1, 12, 20, 23) indicate that metabolic disturbances are induced by this chemical.

Most investigations concerning the effect of chemicals on virus multiplication, and the physiological response of plants to virus infection, have been done with detached leaves or leaf discs. It is felt that the predominant catabolic processes occurring in such materials serve only to complicate further already intricate biochemical mechanisms. This paper reports the occurrence and nature of certain biochemical changes produced by thiouracil in virus-infected and noninfected tobacco plants grown under controlled nutrient conditions.

MATERIALS AND METHODS

The selection of a suitable host-virus combination for this investigation was a prime importance. It has been pointed out that the host plant is important in determining the effectiveness of a specific chemical in inhibiting virus multiplication (1). As indicated above, thiouracil has been effective in tobacco as an inhibitor of all viruses investigated. Thus, it was apparent that tobacco (*Nicotiana tabacum* L.) would be the best choice as an experimental host. The variety Vamorr 48 was selected because of its excellent growth habit, and because its hypersensitive reaction to TMV infection would make possible the detection of chance contamination by TMV. The seed was sown in washed white quartz sand. Uniform seedlings were selected after 6 weeks and were lined out in flats of sand. The plants were watered daily with one-quarter strength nutrient solution. When the plants were about 11 weeks old they were selected for uniformity a second time and were transferred into containers of one-half strength nutrient solution. This solution was replaced with full strength solution one week later, and an additional selection for uniformity was made. The nutrient solution (39) contained the following macronutrient salts: 0.001M potassium dihydrogen phosphate, 0.002M potassium sulfate, 0.002M magnesium sulfate heptahydrate, 0.005M calcium nitrate tetrahydrate; and micronutrient elements: 0.25 p.p.m. of manganese as manganous sulfate monohydrate, 0.10 p.p.m. of zinc as zinc sulfate heptahydrate, 0.10 p.p.m.

of boron as boric acid, 0.10 p.p.m. of copper as cupric sulfate pentahydrate, 0.10 p.p.m. of molybdenum as sodium molybdate dihydrate, and 1.00 p.p.m. of iron as ferric disodium ethylenediaminetetraacetate.

The containers employed were square 10-liter polyethylene pans. The sides were covered with aluminum foil to to eliminate light. Each container was fitted with a paraffinized transite cover, which had 8 holes to accommodate plants and 1 hole for a capillary aeration tube.

When the plants were 13 weeks old, they were transferred to fresh nutrient solution containing thiouracil. The treatments used were 0, 3, and 5 p.p.m. of thiouracil (0, $2.3 \times 10^{-5} M$, and $3.9 \times 10^{-5} M$) in both infected and noninfected series. This resulted in a total of six treatments, each of which was replicated by two containers of 8 plants each. Thus, there were 16 plants per treatment.

Cucumber mosaic virus (CMV) was selected for this investigation because preliminary experiments have shown that thiouracil treatment reduces the amount of this virus in infected tobacco plants. An equally important factor was the desire to determine biochemical changes induced in tobacco by infection with CMV. The CMV strain employed was originally isolated from a cucumber planting in Rock Island, Illinois. It is lethal to cucumbers, produces a severe "fern-leaf" in tomato, and causes a yellow mottle with some leaf distortion in Turkish tobacco. Inoculum was prepared from infected Vamorr 48 stock plants by grinding leaf tissue in $0.1 M$ phosphate buffer, pH 7.0, and straining the brei through two layers of cheesecloth to remove large particulate matter. The plants in the virus series were inoculated two days after thiouracil treatment was initiated. The inoculum was brushed (32) onto the two lower leaves of each plant. Comparable leaves in the nonvirus series were treated in a like manner with buffer only. Symptoms were apparent on the terminal foliage of the infected plants in 0 p.p.m. of thiouracil 3 days following inoculation.

All plants were harvested 7 days after inoculation (9 days after initiation of thiouracil treatment). The inoculated leaves were not included in the sampling. The plants from each container were removed and separated into leaf blades, stems, petioles and midveins, and roots and were weighed. Leaves were cut into pieces approximately 1 inch square and thoroughly mixed. Suitable aliquots were weighed and homogenized in four times their weight of deionized water for one minute at 45,000 r.p.m. in a VirTis "45" Homogenizer. Aliquots of the homogenate were taken by pipette and were analyzed immediately, or after freezing in shell vials and storage at $-20°$ C.

Virus assay was made by inoculating the primary leaves of 9-day-old Black cowpeas (*Vigna sinensis* L.) previously placed in the dark for 24 hours, and then counting the local lesions produced after 2 days in the greenhouse. Ten-milliliter aliquots of fresh homogenate were diluted 1:1 with 0.1M phosphate buffer, pH 7.0, and stored at 4° C. for 24 hours. The cowpeas were inoculated using a ground-glass spatula and carborundum (600 mesh), and the leaves were rinsed with tap water following application of each inoculum. Comparisons were made between the inocula of replications within a treatment and between treatments. Each inoculum was applied to a total of 12 leaves on 12 different plants.

Amino acids, amides, and the nonketo organic acids were extracted with 80 per cent ethanol using 150 milliliters of fresh homogenate which represented 30 grams fresh weight of tissue. Sufficient absolute ethanol was added to the homogenate to result in a final ethanol concentration of 80 per cent. The ethanol extract was separated from the residue by centrifugation and the residue re-extracted four times with 80 per cent ethanol. The extracts were combined and divided into two equal aliquots. One aliquot was subdivided into two equal portions which were used for duplicate determinations of organic acids by the ion-exchange resin chromatography method of Palmer (26) as modified by Staples (31). The remaining aliquot was taken to dryness in a stream of air and the residue taken up in a mixture of equal volumes of chloroform and water. The resulting emulsion was separated by centrifugation and the aqueous phase used for the quantitative determination of free amino acids and amides by the paper chromatographic method of Porter, Margolis, and Sharp (28).

Determination of amino acids of bulk proteins was carried out with lyophilized residue remaining after extraction with 80 per cent ethanol. The residue was ground in a Wiley mill to pass an 80-mesh screen. Samples were extracted for 16 hours with a refluxing water-alcohol mixture (36). Two-hundred milligram samples were placed in ampules with 15 ml. of 6N HCl, and were autoclaved at 15 pounds of pressure for 16 hours. The resulting hydrolyzates were filtered through glass wool and Whatman No. 1 paper, and the HCl removed by repeated evaporation to dryness on the steam bath. The residue was taken up in 2 ml. of 10 per cent isopropanol and this solution was used for amino acid determinations. Methionine, valine, and isoleucine were determined following separation by one-dimensional descending chromatography in 2-butanol:3 per cent ammonia (4, p. 423). It was possible to determine leucine by difference since a value for total leucines was obtained following two-dimensional chromatography. Corrections were not applied for losses incurred during hydrolysis.

Protein nitrogen was determined on one-gram fresh weight aliquots by the semimicro Kjeldahl procedure (25, pp. 805–806) after three extractions with hot 80 per cent ethanol (39). Ammonia was determined by the method of Vickery and Meiss (35).

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) were extracted differentially from one-gram fresh weight aliquots by the perchloric acid method of Howard (13). Nucleic acid phosphorus was determined in the perchloric acid digests by the reduced molybdate method of Pons *et al.* (27).

Inorganic and total phosphorus were determined (27) on one-gram fresh weight aliquots of tissue. Organic phosphorus and nonnucleic acid organic phosphorus were calculated by difference.

Dry weight determinations were made on one-gram fresh weight aliquots dried at 110° C. in tared weighing bottles.

RESULTS

Analytical results are presented on the basis of the weight of total leaf blade tissue per plant. The experimental plants were considered as individual physiological entities that had more or less equivalent numbers of leaves and of cells at the initiation of the experiment. Therefore it seemed reasonable to evaluate the biochemical changes produced by virus infection or by thiouracil treatment on this basis. To have expressed these changes on the basis of fresh or of dry weight of leaf tissue was not considered justified since, depending upon the treatment, each such unit of weight would represent a different proportion of the entire physiological unit.

Symptom development. Figure 1 A shows a general view of plants in culture solutions at the initiation of treatments and Figure 1 B just prior to harvest. Noninfected plants grown at 0 p.p.m. thiouracil were large, well developed, and had 8 or 9 leaves per plant at the time of harvest. Roots were well developed, white, and secondary root development was excellent. Infected plants in this treatment were about the same size and had the same general appearance as noted above. However, young leaves exhibited moderate wilting, occasional necrotic areas along the veins, and a very mild diffuse mottle (Fig. 2 A).

Noninfected plants grown at a level of 3 p.p.m. of thiouracil each had 9 leaves. The upper 5 leaves showed moderate to severe interveinal chlorosis beginning at the base of the leaves and extending distally. The leaves remained green in color along the veins. Young leaves were moderately cupped and strapped and exhibited some tearing of laminae. Secondary root development was poor and roots were slightly discolored. Infected plants in this thiouracil treatment were similar in general appearance to noninfected plants except that most of the plants had 8 leaves, showed moderate to severe veinal necrosis of the upper 4 leaves (Fig. 2 B) and necrotic lesions on lower inoculated leaves (Fig. 2 D).

Noninfected plants grown at a level of 5 p.p.m. of thiouracil had 7 leaves each and exhibited more severe chlorosis than did plants grown at 3 p.p.m. of thiouracil. Infected plants each had 7 or 8 leaves and exhibited severe veinal necrosis (Fig. 2 C). Inoculated leaves had developed severe necrotic spotting and were desiccated (Fig. 2 D). Root development on

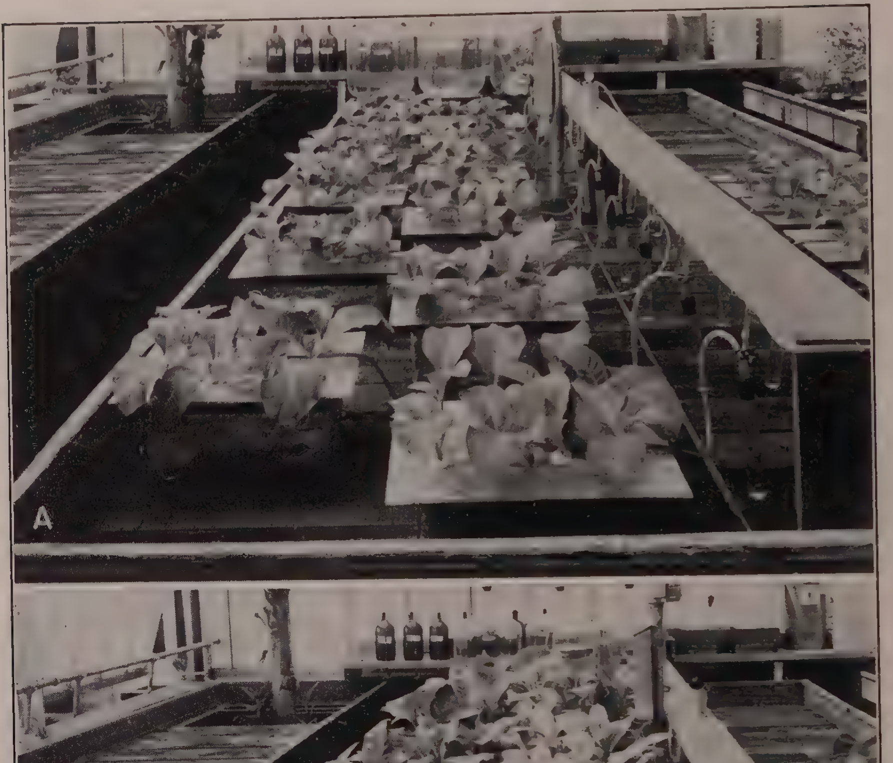

FIGURE 1. Vamorr 48 tobacco plants in culture solutions at (A) initiation of experiment, and (B) just prior to harvest.

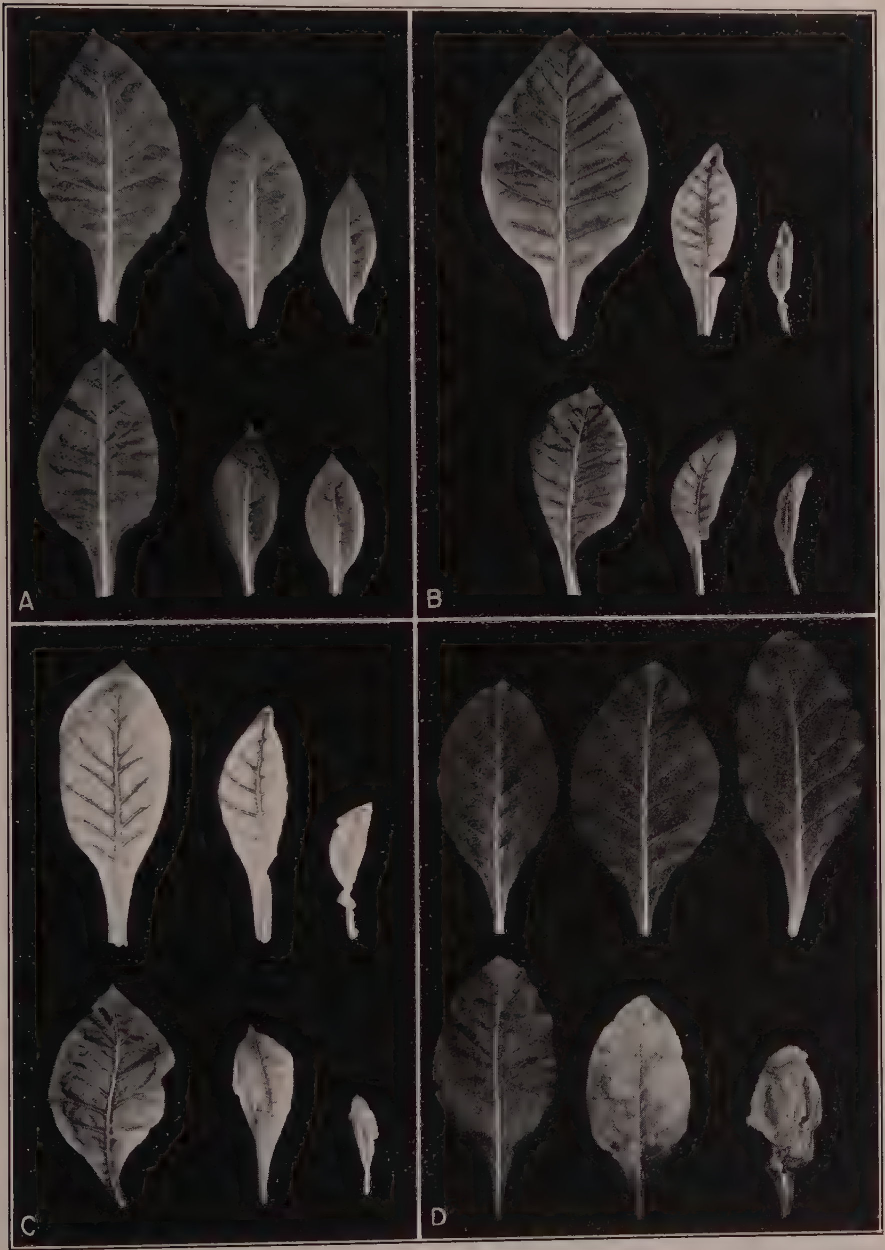

FIGURE 2. Condition of young leaves from Vamorr 48 tobacco supplied with 0 (A), 3 (B), and 5 (C) p.p.m. of thiouracil. In each group leaves from noninfected plants are above and CMV-infected below. (D) Condition of inoculated leaves (lower) from plants receiving 0, 3, and 5 p.p.m. of thiouracil with healthy controls (above).

both infected and noninfected plants was essentially the same as described for plants grown at 3 p.p.m. of thiouracil.

Infected plants in the 3 and 5 p.p.m. of thiouracil treatments exhibited less severe chlorosis than did comparable noninfected plants (Fig. 2 B and C).

Weights. Fresh weight of noninfected plants was lowered with increasing concentrations of thiouracil, with the greatest difference in fresh weight occurring between 3 and 5 p.p.m. of thiouracil. Changes in fresh weight induced by thiouracil treatment in infected plants were greater than in noninfected plants, but in this series the most pronounced effect was between the 0 and 3 p.p.m. treatments. Fresh weights of infected plants were nearly equal at 3 and 5 p.p.m. Virus infection alone had little effect on fresh weight, and the interaction of virus and thiouracil resulted in lower fresh weights (Fig. 3 A).

Differences in dry weights follow the same pattern in noninfected and in infected plants as was noted for fresh weight. Infected plants had a greater dry weight than did noninfected ones in nutrient solution without thiouracil. The combination of virus infection and of 3 p.p.m. of thiouracil resulted in a lower dry weight than did this concentration of thiouracil alone. The dry weights of infected and noninfected leaves were equal at 5 p.p.m. of thiouracil (Fig. 3 B).

Figure 3 C shows that the percentage of dry weight of leaves was less in noninfected than in infected plants at all concentrations of thiouracil. Greatest effects were apparent at 0 and at 5 p.p.m.

Weight of the lyophilized residue after 80 per cent ethanol extraction showed the same pattern as noted for total dry weight values (Fig. 3 D). Fifty to 60 per cent of the total dry material was extracted by 80 per cent ethanol. The relative proportion of alcohol-soluble and insoluble materials did not differ appreciably in the various treatments.

Virus titer. A relative measure of the total active virus present in each infected plant, expressed as local lesions per plant (Fig. 3 E), was attained by correcting the virus titer obtained for each treatment for the differences in plant size produced by 0, 3, and 5 p.p.m. of thiouracil. Virus titer was reduced by thiouracil, with the greatest difference evident between 0 and 3 p.p.m. Treatment with 3 p.p.m. of thiouracil reduced the relative titer to about 27 per cent, and with 5 p.p.m. to about 12 per cent of that of infected plants at 0 p.p.m.

Nonketo organic acids. Column chromatography of the organic acids indicated the presence of succinic, malic, citric, and two or more unidentified organic acids. The unidentified organic acids were eluted from the column together but were resolved by paper chromatography. Both malic and citric acids were isolated and identified by infrared spectra, by co-

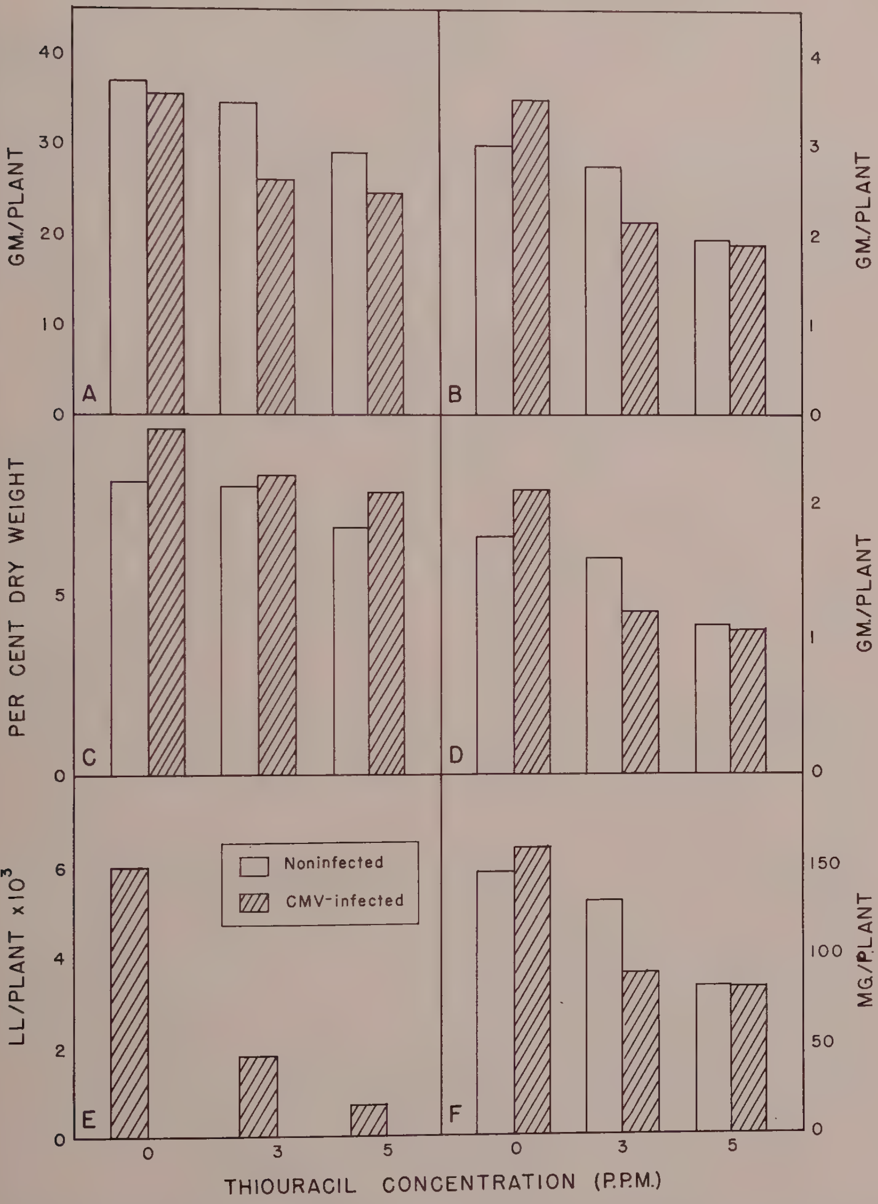

FIGURE 3. (A) Fresh weight, (B) dry weight, (C) per cent dry weight, (D) dry weight of alcohol-insoluble residue, (E) virus titer as local lesions, and (F) protein nitrogen of leaves of noninfected and infected tobacco plants supplied nutrient solutions with different concentrations of thiouracil.

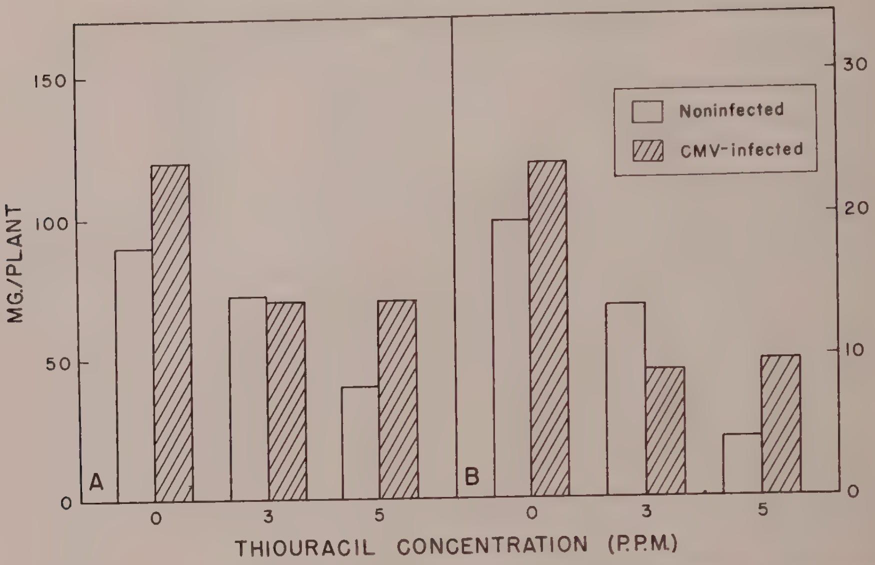

FIGURE 4. Concentration of (A) malic acid and (B) citric acid in leaves of noninfected and infected tobacco plants supplied nutrient solutions with different concentrations of thiouracil.

chromatography on paper with authentic samples, and by melting point determinations.

Succinic acid was not isolated or determined quantitatively since it was present in very small quantities.

The concentration of malic and citric acids was lower in both noninfected and infected leaf tissues of plants grown with thiouracil (Fig. 4 A and B). In the infected series, the levels of malic and citric acids were similar at 3 and 5 p.p.m. of thiouracil. Infected plants had a higher concentration of both acids than did noninfected plants at 0 and 5 p.p.m. of thiouracil. The levels of malic acid were equal in noninfected and in infected plants at 3 p.p.m. of thiouracil, while citric acid was higher in noninfected plants at this thiouracil concentration.

Nitrogen. Increasing concentrations of thiouracil in both noninfected and infected leaves resulted in lower concentrations of protein nitrogen. At 3 p.p.m. of thiouracil, the effect was more pronounced in infected than in noninfected tissues. At 5 p.p.m., both noninfected and infected tissues contained about the same level of protein nitrogen (Fig. 3 F).

There was an increasing concentration of total free amino and amide nitrogen with increasing thiouracil level. The pattern was similar in both noninfected and infected tissues, but on a comparative basis, infected tissues accumulated a slightly higher concentration at 0 and 3 p.p.m. of thiouracil. At 5 p.p.m., noninfected tissues had a higher level than did infected tissues. The observed increases from 0 to 5 p.p.m. of thiouracil are due in large part to the higher level of amide nitrogen, especially of glutamine (Table I).

Changes in individual free amino acids and amides are shown in Figure 5. No basic pattern is followed by all amino acids either as affected by thiouracil treatment, by virus infection, or by a combination of the two. The high levels of free amino acid and amide nitrogen in leaf tissues of noninfected plants treated with thiouracil were contributed primarily by proline, alanine, threonine, valine, arginine, aspartic acid, glutamic acid, leucine(s), lysine, glutamine, and asparagine. In infected leaf tissues it was contributed primarily by serine, threonine, arginine, aspartic acid, glutamic acid, leucine(s), lysine, glutamine, and asparagine.

TABLE I

EFFECT OF THIOURACIL IN NUTRIENT SOLUTION ON THE CONCENTRATION OF ALCOHOL-SOLUBLE AND ALCOHOL-INSOLUBLE NITROGEN FRACTIONS OF LEAVES OF NONINFECTED AND CMV-INFECTED TOBACCO PLANTS

Constituent measured	Concn. (mg./plant) in plants exposed to thiouracil at different levels					
	0 p.p.m.		3 p.p.m.		5 p.p.m.	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
Alcohol-soluble						
Amino-N	3.0	3.7	3.5	3.6	4.5	4.6
Asparagine-N	0.3	0.4	1.0	2.6	3.3	3.4
Glutamine-N	1.7	2.2	8.8	7.9	12.4	9.9
Ammonia-N	0.7	1.0	0.9	0.9	0.7	0.6
Total soluble-N	5.7	7.3	14.2	15.0	20.9	18.5
Alcohol-insoluble						
Amino-N	101.8	101.3	88.5	62.5	56.5	36.6
Ammonia-N	11.6	15.0	13.1	9.9	9.1	4.9
Total insoluble-N	113.4	116.3	101.6	72.4	65.6	41.5
Total	119.1	123.6	115.8	87.4	86.5	60.0

Results of serine and proline analyses each indicate an inverse relationship between noninfected and infected plants at each thiouracil level although the patterns of the two amino acids are reversed. A comparison between results for γ-aminobutyric and glutamic acids shows an inverse relationship between the two acids, i.e., when the level of one acid became lower, there was a comparable rise in the level of the other.

The ammonia content of noninfected plants was slightly higher at 3 p.p.m. than at 0 and 5 p.p.m. of thiouracil. Infected plants exhibited lower concentrations with increasing thiouracil concentrations. There was a higher level of ammonia present in infected than in noninfected leaves at

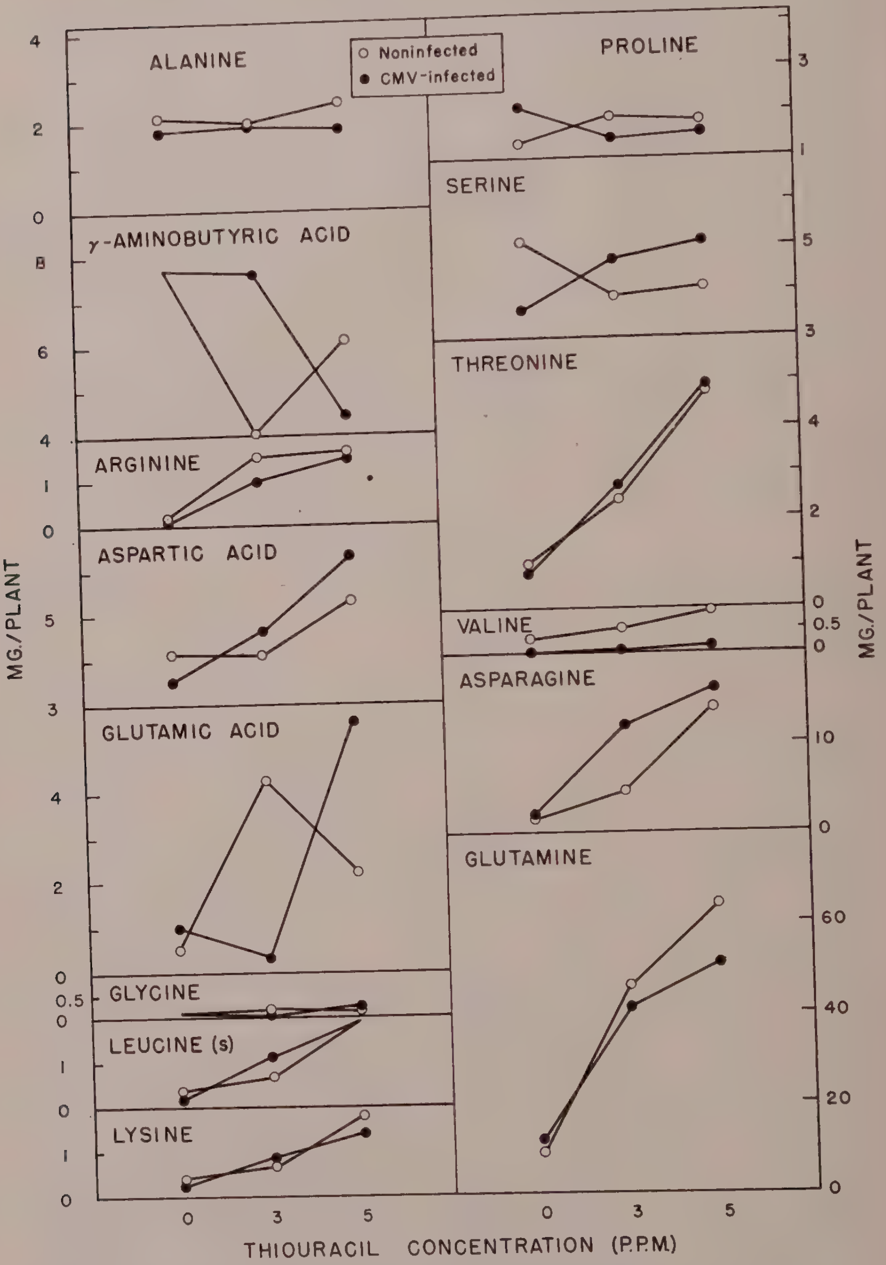

FIGURE 5. Concentration of alcohol-soluble amino acids and amides in leaves of noninfected and infected tobacco plants supplied nutrient solutions with different concentrations of thiouracil.

0 p.p.m.; while the quantities were nearly equivalent at 3 and 5 p.p.m. (Table I).

Results of the quantitative determination of amino acids and ammonia liberated by $6N$ HCl hydrolysis are shown in Table II. Comparisons of the molar ratios of amino acids indicate differences in the constitution of the bulk proteins as affected by thiouracil treatment and by virus infection.

Phosphorus. Figure 6 A shows that the level of total phosphorus in noninfected and infected plants was reduced by thiouracil. The greatest difference in noninfected leaves occurred between 3 and 5 p.p.m., while in infected leaves, it occurred between 0 and 3 p.p.m.

Inorganic phosphorus was not greatly affected by thiouracil in leaves of noninfected plants. However, the level in infected leaves grown with 3 and 5 p.p.m. of thiouracil was lower than infected leaves not supplied with thiouracil. Virus infection alone had little effect on the inorganic phosphorus level (Fig. 6 B).

Increasing concentrations of thiouracil resulted in lowered levels of organic phosphorus in both noninfected and infected leaf tissues. Concentrations of 3 and 5 p.p.m. of thiouracil in noninfected plants resulted in levels that were 26 and 53 per cent and in infected plants of 53 and 66 per cent lower than that of leaf tissues of plants grown without thiouracil. In plants grown without thiouracil, virus infection had no effect on the organic phosphorus level (Fig. 6 C).

Both virus infection and thiouracil treatments resulted in low levels of nonnucleic acid organic phosphorus. At both concentrations of thiouracil, the levels of this phosphorus fraction in infected plants were lowered to a much greater extent than in noninfected plants (Fig. 6 D).

Both RNA-P and DNA-P are lowered with increasing thiouracil levels. Infected plants grown without thiouracil had a higher level of both types of nucleic acid phosphorus than did noninfected plants (Fig. 6 E and F).

Table III presents the described analytical results as the percentage differences produced by thiouracil, by virus infection, and by their interaction in the experimental tobacco plants. Values for noninfected plants grown without thiouracil are expressed as 100 per cent. Differences in virus titer, however, are based on infected plants grown without thiouracil.

DISCUSSION

It is apparent from the results that major changes were induced in the tobacco plants by infection with CMV and in both noninfected and infected plants by treatment with thiouracil. The noticeably less severe chlorotic symptoms in infected than in noninfected plants treated with thiouracil may have been due to the incorporation of a large proportion of the absorbed thiouracil into noninfective virus molecules; thus, there was not sufficient free thiouracil present to induce as severe chlorosis as

TABLE II

AMINO ACID COMPOSITION OF THE BULK LEAF PROTEINS OF NONINFECTED AND CMV-INFECTED VAMORR 48 TOBACCO TREATED WITH 0, 3, AND 5 P.P.M. OF THIOURACIL. VALUES EXPRESSED ON A GRAM OF ALCOHOL-INSOLUBLE NITROGEN BASIS. MOLAR RATIOS CALCULATED TO NEAREST WHOLE NUMBERS

Amino acid	Ratios and concentration of amino acids (mg./g. of alcohol-insoluble N)																	
	0 p.p.m. Thiouracil						3 p.p.m. Thiouracil						5 p.p.m. Thiouracil					
	Noninfected			Infected			Noninfected			Infected			Noninfected			Infected		
	Mg.	Mg. N	Molar ratios	Mg.	Mg. N	Molar ratios	Mg.	Mg. N	Molar ratios	Mg.	Mg. N	Molar ratios	Mg.	Mg. N	Molar ratios	Mg.	Mg. N	Molar ratios
Alanine	358	56.2	16	317	49.8	10	342	53.7	12	319	50.1	11	370	58.1	19	317	49.8	12
Arginine	337	108.5	8	292	94.0	5	291	93.7	5	268	86.3	5	281	90.5	8	250	80.5	5
Aspartic acid	338	35.5	10	337	35.4	7	308	32.3	7	306	32.1	7	385	40.4	13	355	37.3	9
Cystine	86	7.1	1	120	10.0	1	106	8.8	1	108	9.0	1	72	6.0	1	102	8.5	1
Glutamic acid	391	37.1	10	313	29.7	6	411	39.0	9	352	33.4	8	400	38.0	13	326	31.0	7
Glycine	250	46.8	13	189	35.3	7	207	38.7	9	182	34.0	8	242	45.3	15	169	31.6	8
Histidine	213	57.7	8	146	39.6	3	169	45.7	4	162	43.9	3	162	43.8	5	137	37.2	3
Isoleucine	330	35.3	10	415	44.4	9	290	31.0	5	305	32.6	7	478	51.2	17	304	32.5	8
Leucine	880	94.5	26	661	70.7	14	812	86.9	14	762	81.5	18	580	62.1	15	736	78.8	19
Lysine	480	92.2	13	391	75.1	8	524	100.6	11	486	93.3	10	562	107.9	18	479	92.0	11
Methionine	133	12.5	3	162	15.2	3	104	9.8	2	130	12.2	3	125	11.8	4	147	13.8	3
Phenylalanine	942	80.1	22	542	46.1	9	632	53.7	12	598	50.8	11	529	45.0	15	868	74.0	18
Proline	360	43.9	12	458	55.9	11	331	40.4	9	409	49.9	11	373	45.5	15	525	64.1	15
Serine	162	21.5	6	162	21.5	4	161	21.4	5	168	22.3	5	183	24.3	8	155	20.6	5
Threonine	222	26.2	7	192	22.7	5	205	24.2	6	194	22.9	5	222	26.2	9	186	21.9	5
Tyrosine	280	21.6	6	192	14.8	3	182	14.0	3	208	16.0	4	272	20.9	7	235	18.1	4
Valine	448	53.8	15	409	49.1	10	418	50.2	11	441	52.9	12	453	54.4	18	392	47.0	11
Ammonia from hydrolysis		93.3			103.4			109.0			112.7			123.6			98.8	
Total	6210	923.8		4981	812.7		5493	744.1		5398	835.9		5689	895.0		5683	837.5	

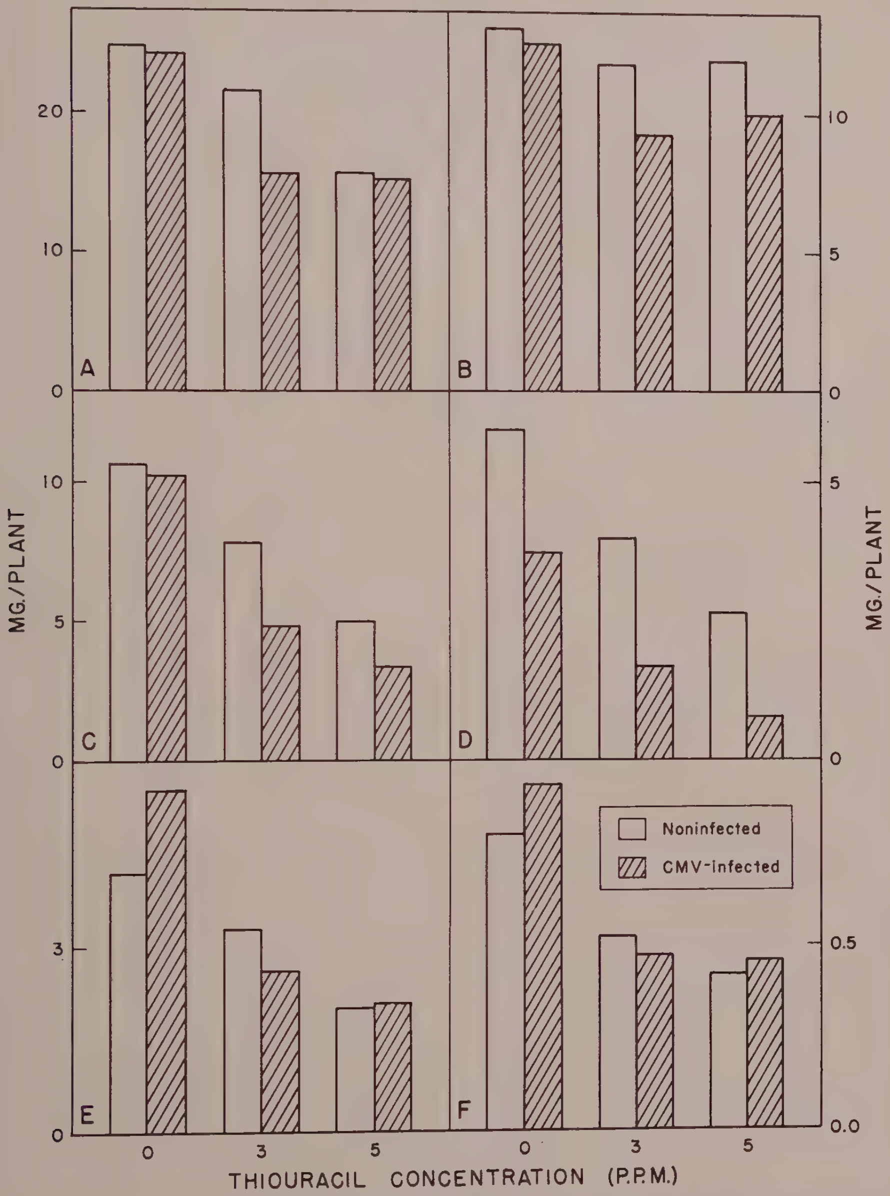

FIGURE 6. Concentration of (A) total phosphorus, (B) inorganic phosphorus, (C) organic phosphorus, (D) nonnucleic acid organic phosphorus, (E) RNA-phosphorus, and (F) DNA-phosphorus in leaves of noninfected and infected tobacco plants supplied nutrient solutions with different concentrations of thiouracil.

TABLE III

Percentage Differences in Weights, Virus Titer, Nonketo Organic Acids, and Nitrogen and Phosphorus Fractions of Noninfected and CMV-infected Leaves of Tobacco Plants Supplied with Nutrient Solutions with Different Concentrations of Thiouracil. Values for Noninfected Plants Grown without Thiouracil Are Expressed as 100 Per Cent. Virus Titer Based on Infected Plants Grown without Thiouracil

	Percentage differences					
	Noninfected			Infected		
	Thiouracil concn. (p.p.m.)			Thiouracil concn. (p.p.m.)		
	0	3	5	0	3	5
Weights						
Fresh	100	93	78	95	70	66
Dry	100	92	66	117	71	64
Alcohol-insoluble residue	100	93	62	123	69	58
Virus titer	—	—	—	100	27	12
Nonketo organic acids						
Malic	100	80	49	133	78	78
Citric	100	70	22	121	46	49
Nitrogen						
Alcohol-soluble	100	249	367	128	264	325
Alcohol-insoluble	100	90	58	103	64	37
Total alcohol-soluble and insoluble	100	97	73	104	73	50
Phosphorus						
Total	100	87	64	98	63	62
Inorganic	100	89	91	96	70	76
Organic	100	73	47	96	45	31
Nonnucleic acid organic	100	67	45	63	28	14
RNA	100	79	48	132	64	50
DNA	100	66	53	118	59	59

was apparent in noninfected plants. It is also possible, of course, that the uptake of thiouracil by roots of infected plants was restricted, although no unusual inhibition of root development was apparent.

The greater dry weight of infected plants is not unusual and similar observations have been made for other virus-infected hosts (40). Results of the present investigation indicate that this condition is due to a virus-induced stimulation of host metabolism that is reflected by increase in protein-, alcohol-soluble amino-, amide-, and ammonia-nitrogen, RNA-P, DNA-P, and malic and citric acids.

The increase in RNA-P may have been partially contributed by virus, but, because of the magnitude of the increase, it appears that there was a stimulation of RNA synthesis induced by infection. The higher DNA-P in infected plants strongly suggests a stimulation in cellular division resulting in more total cells.

Probably the most interesting effect of CMV-infection was the reduction in nonnucleic acid organic phosphorus. Since the levels of inorganic and total phosphorus were only slightly reduced, virus infection did not appear to affect materially the uptake of phosphorus from the nutrient medium. Therefore, it seems likely that infection was associated with a stimulated utilization of organic phosphorus compounds such as phosphorylated sugars, nucleotides, ATP, etc. as energy sources or component parts in the synthesis of more complex molecules such as nucleic acids, proteins, and nucleoproteins.

In the thiouracil series, reduced dry weights of noninfected and infected plants with increases in thiouracil concentration emphasize its toxicity to processes other than those associated with chlorophyll synthesis.

Although the data show that total amino-, amide-, and ammonia-nitrogen (soluble and insoluble) were lowered by thiouracil treatment (Table I), it is significant that the portion contributed by the soluble amino acids and amides was actually increased. It is apparent, then, that the effect of thiouracil upon nitrogen metabolism was reflected by a block in the synthesis of bulk proteins of the leaf; however, a block of the same magnitude did not occur in the synthesis of amino acids and amides. It is suggested that this effect exists because of an aberration in nucleic acid metabolism due to the antimetabolic activity of thiouracil with uracil. This is in agreement with the well-established relationship between nucleic acid metabolism and protein synthesis (5), and more specifically with the recent work of Kessler (17) who found that thiouracil overcame the stimulatory effect of uracil on RNA and protein synthesis in leaves of the olive and grape.

Observed differences in molar ratios of amino acids from hydrolyzed leaf proteins in each of the treatments could reflect the synthesis of new species of proteins. Infection with TMV results in the synthesis of new and different proteins in the tobacco leaf (2, 3, 10, 11, 14, 33, 34), and the proper concentrations of thiouracil may induce an increase or a decrease in certain groups of proteins (10). Inhibition of synthesis of TMV or other viruses by thiouracil indicates that in all probability the molar ratios of protein amino acids would be changed.

The lowered levels of malic and citric acids associated with thiouracil treatments probably result from a partial block in the formation of metabolites feeding into the Krebs cycle or from rapid conversion of keto acids to amino acids with their subsequent interconversion.

The occurrence in yeast of uridine diphosphate glucose serving as a coenzyme in the conversion of galactose-1-phosphate to glucose-1-phosphate (6, 7), and the reports of high energy uridine di- and triphosphates (16, 30), suggests the possibility of a similar antagonistic effect by thiouracil in systems other than those mentioned earlier. Thus, interference with high energy transfer in these plants could result in nonspecific changes in most or all biochemical components. The observed decrease in nonnucleic acid organic phosphorus could have influenced the incorporation of amino acids into proteins since Webster (37) has shown the need of high-energy phosphate for this reaction. He also found that thiouracil did not inhibit incorporation of glutamic acid into proteins (38). This does not necessarily mean, however, that thiouracil will not inhibit protein synthesis since it may inhibit the incorporation of other amino acids and thus effectively reduce protein synthesis.

The present data are in accord with the conclusion that the reduction by thiouracil of infective virus particles in the experimental plants was due to: (a) the incorporation of thiouracil into normal and virus nucleic acids resulting in their qualitative change and a quantitative reduction; and (b) an inhibition in virus protein synthesis due to a lack of normal nucleic acids. These conclusions are supported by the findings that thiouracil is incorporated into nucleic acids (15, 19, 21) and that nucleic acids play an important role in protein synthesis (5, 17).

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Note

APPARATUS FOR PHOTOGRAPHING FLUORESCENT CHROMATOGRAMS

R. Mavrodineanu and Myron C. Ledbetter

The use of fluorescence for the identification and measurement of substances separated on paper chromatograms is well known (1, pp. 32–33, 56–57; 5; 8; 9, p. 101). In this note a description is given of an instrument constructed to obtain photographic records of paper chromatograms by transparency in color and black and white in emission. The extent of ultraviolet (u.v.) absorption can also be recorded on the same instrument.

This apparatus was used in connection with studies of unstable plant pigments (like chlorophyll, pheophytin, xanthophyll, and carotenoids). It also proved to be indispensable for the identification of monofluoroacetic acid in extracts of gifblaar (*Dichapetalum cymosum* Hook.).

DESCRIPTION OF THE APPARATUS

The instrument is constructed in a metal frame paneled with transite to resist heat (Fig. 1). It contains two u.v. lamps, L, of 450 w. each (Hanovia #S 400) mounted in individual aluminum reflectors, R, above which two heat absorbing filters, H, are placed (Corning #4600). The u.v. lamps are powered by individual reactance transformers, T, operated by two switches (out of view in the figure). On top of the enclosure, E, an u.v. transmitting filter, C, is placed. It is made from two $6\frac{1}{2} \times 6\frac{1}{2}$ in. polished Corning #5860 filters mounted together in a metal frame with a useful field of $5\frac{1}{2} \times 11\frac{1}{2}$ in. These filters have a transmission from 300 mμ to 400 mμ with a maximum at 360 mμ and are protected from the heat developed by the u.v. lamps by filters, H, and fan, F. A camera can be placed on the metal arm, A, permitting the photography of chromatograms placed over filters, C.

Two complementary u.v. absorbing filters (Y-1 by Ednalite) transmitting the radiation from 410 mμ (2, pp. 203–213, 323–329) through the visible part of the spectrum are placed in front of the camera lens. It is important to mention here that these filters are nonfluorescent.

To operate the apparatus the lamps are switched on for 15 min.; the chromatograms are placed over the filters, C, and covered with an ordinary glass plate to hold them flat when the photograph is taken. This will give a fluorescent emission picture which can be taken in color or black and

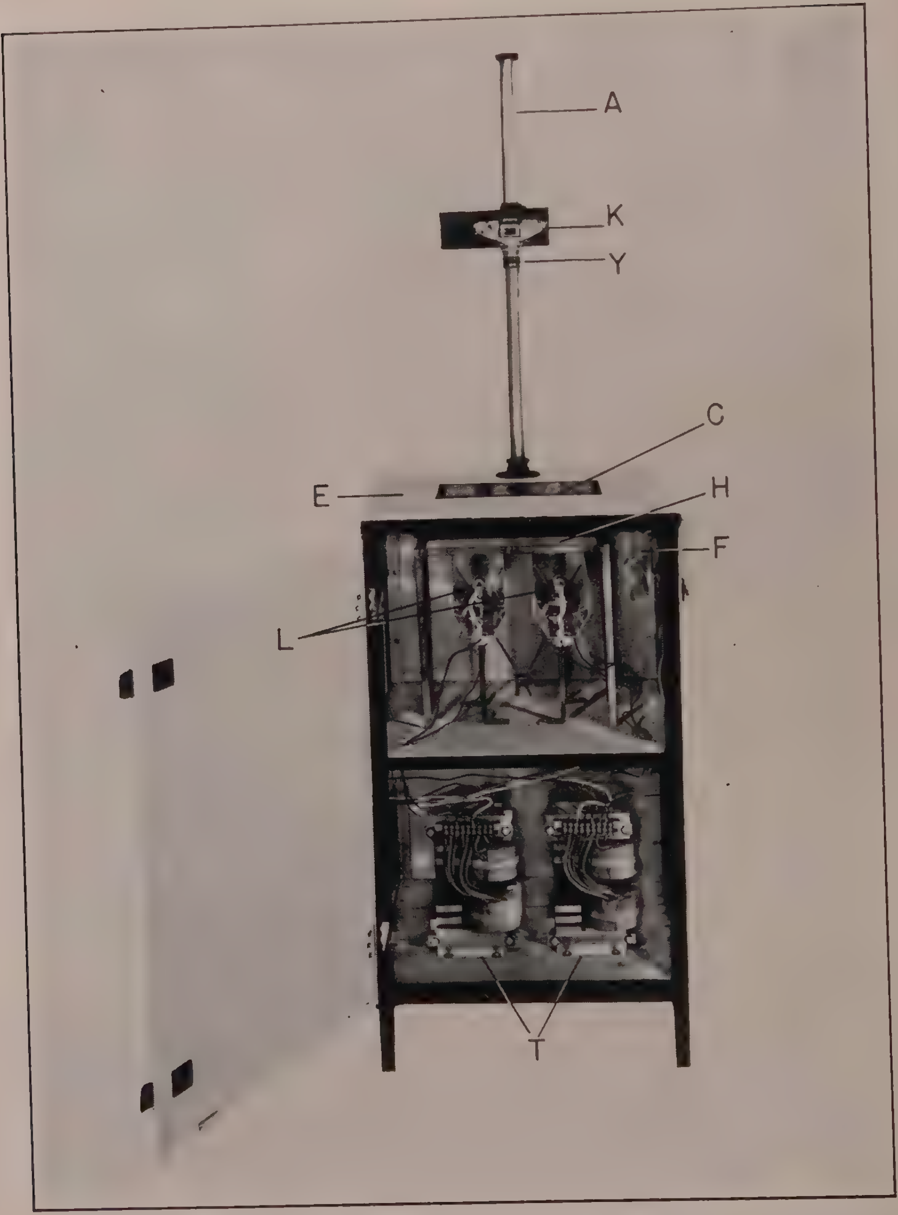

FIGURE 1. Apparatus for photographing fluorescent chromatograms. A, Metal support; C, Corning glass, filter #5860; E, enclosure; F, electric fan; H, heat absorbing filter, Corning #4600; K, Exakta camera 35 mm. with F/3.5 Tessar lens; L, two u.v. lamps, Hanovia #S 400; R, aluminum reflector; T, two reactance transformers; and Y, two filters, Ednalite #Y-1.

white. An u.v. absorption picture can be obtained by placing a fluorescent filter paper above the chromatogram. In this case the u.v. absorbing spots will appear as dark areas on a bright fluorescing background. The fluorescent filter paper is prepared by impregnating a Whatman #1 sheet with a solution of 0.01 per cent Uranin (sodium salt of fluorescein) in water containing 10 per cent glycerine.

Figure 2 A shows an emission fluorescent chromatogram of plant pigments extracted with acetone from tomato (*Lycopersicon esculentum* Mill.) leaves and developed 20 min. in the solvent of Lefort and Signol (3) on oven-dried paper. The paper was cut in a pattern which provided the best separation of constituents (4, 6, 7). The same chromatogram produced the pattern seen in Figure 2 B when taken in u.v. absorption. Xanthophylls which are found between pheophytin a and chlorophyll a do not appear on these prints.

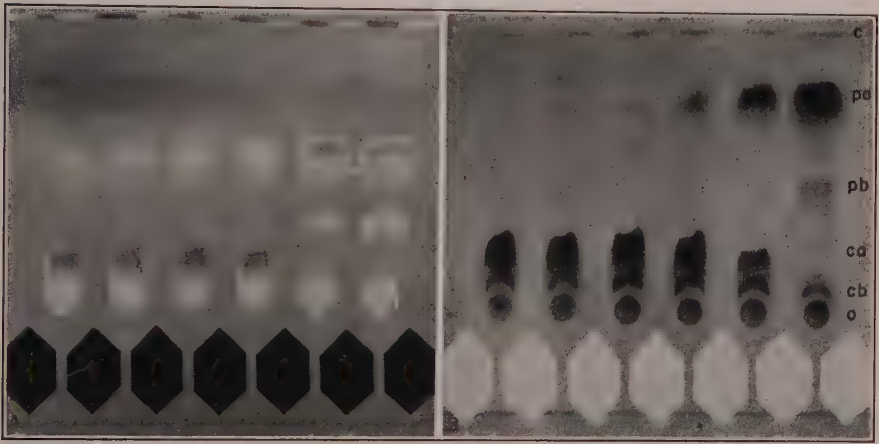

FIGURE 2. Chromatograms of tomato leaf pigments in (A) emission and (B) absorption. (c) Carotenoids; (pa) pheophytin a; (pb) pheophytin b; (ca) chlorophyll a; (cb) chlorophyll b; (o) origin of acetone extract. From left to right are increasing amounts of pheophytins and decreasing amounts of chlorophylls due to increasing amounts of added oxalic acid.

The identification of monofluoroacetic acid in water extracts of gifblaar is based on the fluorescence developed when the chromatogram is sprayed with a 1 per cent alcoholic solution of quinine hydrochloride. In this case the developing solution is made from isopropyl alcohol:tertiary butyl alcohol:benzyl alcohol:water:88 per cent formic acid, 1:1:3:1:0.12. Figure 3 A shows such a chromatogram obtained from monofluoroacetic acid spotted on the paper in various concentrations. This procedure was found to be more sensitive and more specific for the identification of monofluoroacetic acid when compared with chromatograms sprayed with pH

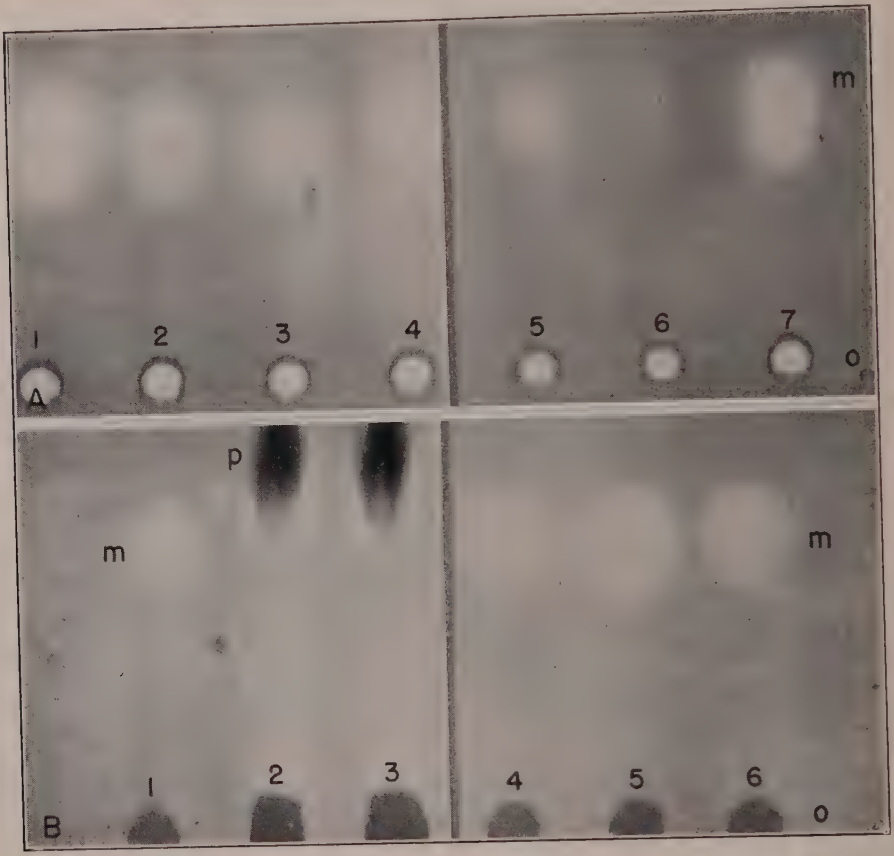

FIGURE 3. Fluorescent chromatograms of monofluoroacetic acid in emission. (A) Fluorescent spot of the acid (m); origin (o). From 1 to 7 different concentrations, respectively, 50, 25, 18, 12, 7.3, and 5 μg. (B) 25 μG. monofluoroacetic acid (m) from standard solutions (1 and 6). Gifblaar water extracts (p) steam-distilled showing the presence of monofluoroacetic acid (2 and 3) and spots of 50 μg. steam-distilled acid (4 and 5).

indicators. Figure 3 B presents the results obtained from chromatographing steam-distilled water extracts of gifblaar leaves and solutions of monofluoroacetic acid. The presence of the acid in the plant extracts is visible at an Rf slightly higher due to the influence of plant pigments.

The exposure times required to obtain these pictures were as follows: for Kodachrome (Daylight or Type F) F/3.5 for 30 sec. in emission; for black and white (Panatomic X) F/8 for 10 sec. in emission and F/11 for 4 sec. in absorption.

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Note

MONOSACCHARIDE COMPONENTS OF AN INDUCED GLYCOSIDE OF TRICHLOROETHYL ALCOHOL FROM DANDELION TOPS

Lawrence P. Miller

In experiments carried out some time ago it was found that various green plants or plant parts readily formed β-glycosides when aglycons or potential aglycons were supplied either in the nutrient medium or in the vapor form (2, 3, 4). An interesting aspect of the results obtained was that the sugar components of the glycosides varied and the particular glycoside formed depended upon the species of plant and on the aglycon. In gladiolus corms, for example (2), β-*o*-chlorophenylgentiobioside was formed from absorbed *o*-chlorophenol but when ethylene chlorohydrin was used only β-2-chloroethyl-D-glucoside could be isolated. The identity of the glycosides isolated was established by comparison with the corresponding synthetic compounds. When these experiments were discontinued a trichloroethylglycoside containing a C_{11} disaccharide had been obtained from dandelion (*Taraxacum officinale* Weber) plants which had been treated with chloral hydrate. This glycoside remained uncharacterized. It was concluded that a C_{11} disaccharide was involved since the chlorine content of the acetate was found to be 15.39 per cent (calcd. 15.29), and a positive test for pentose was obtained with Bial's solution (3).

With the use of paper chromatography the monosaccharide components of this disaccharide have now been identified as glucose and xylose. A 300-mg. sample of the acetate was deacetylated in dry methanol by the use of barium methylate and the barium removed by filtration after titration with sulfuric acid. A portion of the glycoside thus obtained was hydrolyzed by heating for one hour with 1.5 per cent hydrochloric acid. The hydrolyzate was evaporated to dryness several times at room temperature to remove all of the acid and was then taken up in a small volume of water for chromatography. The solvent for the chromatography was prepared by mixing 125 parts of water, 125 parts of *n*-butanol, and 40 parts of pyridine by volume.[1] This mixture separates into two layers, and the top layer was used. The chromatography was carried out by the ascending method on Schleicher and Schüll paper No. 598. The solvent was run over the paper three times with thorough drying of the paper between runs.

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[1] Block, Richard J. Personal communication.

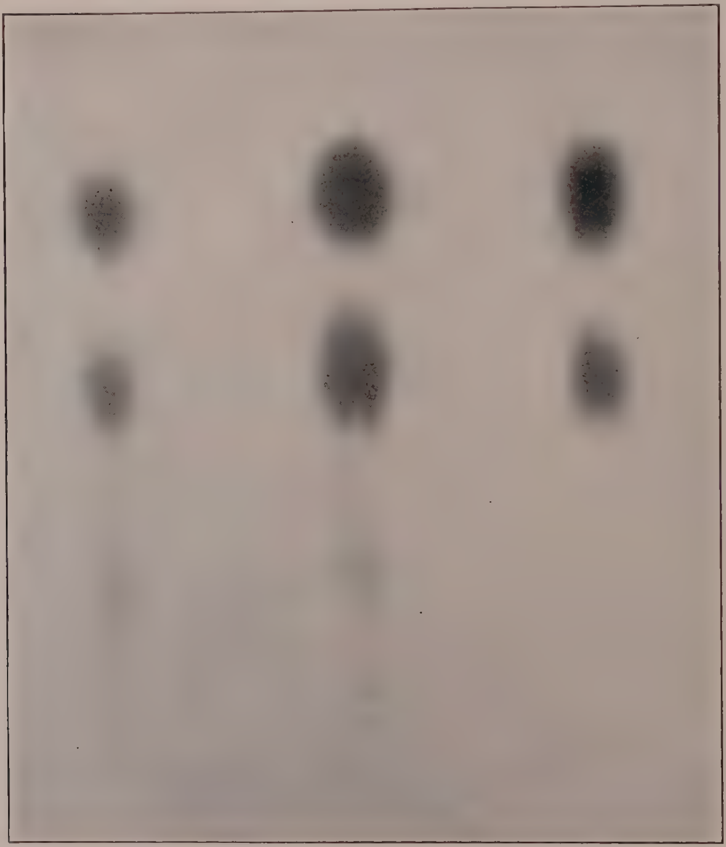

FIGURE 1. Reproduction of paper chromatogram. Left, hydrolyzed unknown C_{11} disaccharide; center, same plus glucose and xylose; right, glucose and xylose.

Spots were located on the paper by bringing out the color with aniline hydrogen phthalate (1, p. 133). By this procedure good separation was obtained for the common monosaccharides. The spot for one of the sugars in the unknown coincided with that for xylose but was also rather close to that of arabinose. Co-chromatography readily showed that the unknown was xylose and not arabinose. A photograph of one of the chromatograms is reproduced in Figure 1. On the left are shown the two spots obtained with the hydrolyzed unknown disaccharide, the spots in the center were obtained when glucose and xylose were added to the unknown, and the right shows the spots obtained with glucose and xylose only.

The above, of course, yields no information as to which of the dissaccharides of glucose and xylose is involved. The most common of the naturally occurring ones is primeverose. This sugar has not been shown to be present in the dandelion; neither has gentiobiose been shown to be a normal constituent of plants which can form large quantities of β-gentiobiosides from aglycons added to the nutrient media. Positive identification of the C_{11} disaccharide must await the preparation of the corresponding synthetic glycoside.

The technical assistance of Miss Ann Doran in these experiments is gratefully acknowledged.

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EFFECTS OF TEMPERATURE AND COMPOSITION ON CRYSTALLITE GROWTH IN BORDEAUX MIXTURES[1]

H. P. BURCHFIELD, JOAN SCHECHTMAN, AND BEATRICE S. MAGDOFF

SUMMARY

The effects of temperature and composition on the rates of crystallite growth in Bordeaux mixtures have been investigated using a method which depends upon the ability of a 10 per cent solution of sucrose in water to dissolve the hydrogel which is formed on the addition of aqueous copper sulfate to a suspension of calcium hydroxide. The relationship between the reciprocal of the absolute temperature and the logarithm of the time required to reduce absorbance by 25 per cent is linear. However, the rate-determining step cannot be a chemical reaction, since the time required for the onset of rapid growth is reduced by seeding. Crystallite growth is retarded by inorganic anions and cations and by polyhydroxy organic compounds, amino acids, and lignosulfonates. The most effective inorganic materials tested for stabilizing the hydrogel are Mn^{++}, Zn^{++}, and $AsO_4^{\equiv}$ while the most effective organic stabilizers tested are coconut milk solids, sucrose, and lignosulfonates in that order. Stability is reduced by dilution and seeding.

The most effective way so far discovered to improve the physical characteristics of the hydrogel for use as an agricultural spray is by reducing the lime content to 40 per cent or less of the amount of $CuSO_4 \cdot 5H_2O$ used in the formulation. Thus 10-4-100 Bordeaux mixture forms microcrystals, which adhere well to foliage even after prolonged aging, while 10-3.2-100 Bordeaux forms even smaller crystallites and in addition possesses outstanding settling characteristics.

INTRODUCTION

Subsequent to the finding (1) that crystallite growth in Bordeaux mixtures could be measured quantitatively by a colorimetric method that depends upon the ability of aqueous sucrose to dissolve the precipitate formed on adding aqueous copper sulfate to a suspension of lime, studies were undertaken to determine the effects of temperature and composition on the stability of the hydrogel with respect to this property. These investigations were given additional impetus by the collateral finding that aged 10-10-100 Bordeaux mixture has almost no ability to adhere to foliage under artificial rain, while the freshly prepared mixtures form spray deposits which are considerably more tenacious than cuprous oxide.

In this discussion the term "rate of crystallite growth" should be interpreted to mean the rate of conversion of the readily solubilized hydrogel to less soluble sphaerocrystals (1), microcrystals, or cupric oxide since the nature of the products formed on aging varies considerably depending on

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[1] This work was sponsored jointly by the United Fruit Co., Boston, Mass., the Phelps Dodge Refining Co., New York, N.Y., and the Standard Fruit and Steamship Co., New Orleans, La., from 1954 to 1957.

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the ratio of copper to lime used in the initial mixture. Because the end-products differ in their solubilities in aqueous sucrose, experimental results are reported in terms of the time required for the onset of rapid growth (t_i), wherever possible, or the time required for the absorbances of the test solutions to decrease by 25 per cent (T25). This latter parameter is empirical but it does not differ appreciably from the per cent conversion ($t_{\frac{1}{4}}$) figures previously reported for 10-10-100 Bordeaux mixture, while its use makes it possible to avoid endpoint readings on a large number of compositions, many of which do not reach equilibrium for several months to a year.

MATERIALS AND METHODS

Chemicals. Samples of commercial lime from eight different sources as well as Marasperse C (calcium lignosulfonate), and Maracarb NC (a mixture of sodium and calcium lignosulfonates) were supplied by the United Fruit Company and samples of commercial copper sulfate by the Phelps Dodge Refining Corporation. Analytical Reagent Grade calcium hydroxide and cupric sulfate pentahydrate were obtained from the Mallinckrodt Chemical Company, casein from the Fisher Scientific Company, and hydrolyzed casein from Nutritional Biochemicals Corp. The inorganic stabilizers that were evaluated were Reagent Grade chemicals purchased from various suppliers. Coconut milk solids were obtained by evaporating the milk from ripe coconuts in a freeze dryer. The yield was 5 per cent of the wet weight.

Method. The chelation test was carried out as described previously (1) except that the medium porosity sintered glass funnels were replaced with coarse grade Mandler diatomaceous filter cylinders, since the former allowed fine particles of gypsum to filter through in the early stages of the experiments. In cases where the amount of lime was reduced, an equivalent amount of sodium hydroxide was added to the chelating solution since it was found that the amount of copper dissolved varied with the total alkalinity of the solution. Where the amount of copper was varied, absorbances were corrected back to values which would have been obtained on a 10-10-100 mixture to provide for uniformity of treatment. Inorganic adjuvants were added to the lime before suspending it in water. Organic adjuvants were dissolved in 10 ml. of water from the lime suspension and added to the Bordeaux 5 minutes after mixing. All experiments were run at 45° C. except when it was desired to study crystallite growth as a function of temperature. Additional details describing the preparation of the Bordeaux mixtures are given in a previous publication[2] (1).

EXPERIMENTAL RESULTS AND DISCUSSION

Effects of temperature and seeding. Bordeaux mixture prepared in proportions equivalent to 10 lb. of hydrated lime and 10 lb. of cupric sulfate pentahydrate per 100 gallons of water changes from a gelatinous precipitate to a crystalloid within a few hours at room temperature. The phase which contains the copper is composed of sphaerocrystals which are probably aggregates of very small crystallites. They appear to be spheres about 5 to 6 μ in diameter. When applied to banana leaves, they have little if any ability to adhere after exposure to artificial rainfall.

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[2] Throughout these investigations concentrations were based on pounds of solids per 100 gallons of water rather than on 100 gallons of mixture.

The rate of conversion of the hydrogel to sphaerocrystals was found to be dependent on temperature. Thus, the induction period (time required for the onset of rapid growth) decreased from 160 minutes at 30° C. to 14 minutes at 50° C. (Table I). Although the relationship between time and temperature was not strictly linear, in general, the useful life of 10-10-100 Bordeaux mixture was halved by each five degree increase in temperature. Under tropical conditions (30° to 35° C.) the mixture would be expected to remain in a usable condition for two to three hours after preparation, providing crystallite growth was not accelerated by seeding and exposure to high ambient temperatures in pipe lines, or retarded by the presence of impurities which stabilize the hydrogel.

TABLE I

EFFECT OF TEMPERATURE ON THE INDUCTION PERIOD AND TIME REQUIRED FOR A 25 PER CENT DECREASE IN ABSORBANCE (T25) OF 10-10-100 BORDEAUX MIXTURE

Temperature (° C.)	Induction period (t_i) (minutes)	T25 in minutes
30	160	200
35	80	110
40	45	55
45	23	29
50	14	19

When 10-10-100 Bordeaux mixtures were pre-aged at room temperature (34° C.) before running chelation tests at 45° C., crystallite growth took place more rapidly than usual, when the T25 was measured from the time of heating. Pre-aging for 26 minutes caused suppression of the induction period while the quarter life (T25) was reduced by about 15 per cent. On a mixture pre-aged for 75 minutes the T25 was reduced by about 40 per cent. Thus, the past history of a sample with respect to age and temperature could be detected by the chelation test.

The time which elapses between mixing the ingredients and the onset of crystallite growth does not represent a period of quiescence, since changes take place which are not detectable by the chelation test. For example, when a 10-10-100 mixture was diluted with 4 volumes of water and placed in a 245-mm. graduated cylinder, the small flocs of hydrogel which it contained aggregated rapidly so that they became visible to the naked eye. Consequently, the settling time was much reduced (Table

TABLE II

EFFECTS OF TIME AFTER MIXING AND TIME AFTER DILUTION ON DISTANCE SETTLED (MM. DURING 5 MINUTES) OF A 10-10-100 BORDEAUX MIXTURE DILUTED WITH 4 VOLUMES OF WATER

Minutes after dilution	Distance settled (mm.) in suspensions held for different periods of time before dilution		
	0 Hr.	1 Hr.	2 Hr.
0	83	62	70
15	79	68	68
30	108	97	64
45	145	128	63

II). When the dilution was made immediately after mixing the Bordeaux, the hydrogel settled a distance of 83 mm. within five minutes. When the material was resuspended at 15-minute intervals, the settling rate increased with time. Thus, 45 minutes after dilution the mixture settled a distance of 145 mm. in 5 minutes compared to 83 mm. in the original test. Therefore, it must be assumed that some change took place after dilution which led to faster settling. On the other hand, when the Bordeaux mixture was aged for two hours *before* making the dilution the settling rate did not increase with subsequent aging, while an intermediate situation occurred when the Bordeaux was allowed to age for one hour before diluting. Since the settling properties of the aged mixture after dilution were distinctly different from those of the freshly prepared mixture some chemical or physical change must have occurred in the structure of the hydrogel during the two-hour aging period.

The occurrence of a chemical reaction was suggested by the finding that a linear relationship was obtained by plotting the logarithm of T25 against the reciprocal of the absolute temperature (Fig. 1). This usually occurs in chemical reactions but is unexpected in crystallite growth. However, the rate-determining step could not be a chemical reaction since crystallite growth was accelerated by seeding (Table III). Addition of sphaerocrystals to freshly prepared 10-10-100 Bordeaux suppressed the induction period entirely and reduced the T25 by about 50 per cent. However, this effect soon reached a limiting value since further decreases in T25 were not obtained when the amount of seed exceeded 5 ml. of aged Bordeaux per 200 ml. of a freshly prepared 10-10-100 mixture. Thus, when an excess of nuclei was provided, rapid crystallite growth began immediately and the T25 was about 15 minutes at 45° C. It would be of interest to determine whether this parameter also obeys the integrated Arrhenius equation.

Effects of impurities and stabilizers. Eight samples of commercial lime obtained from different sources were evaluated in 10-10-100 Bordeaux by the chelation method and significant differences in performance could not

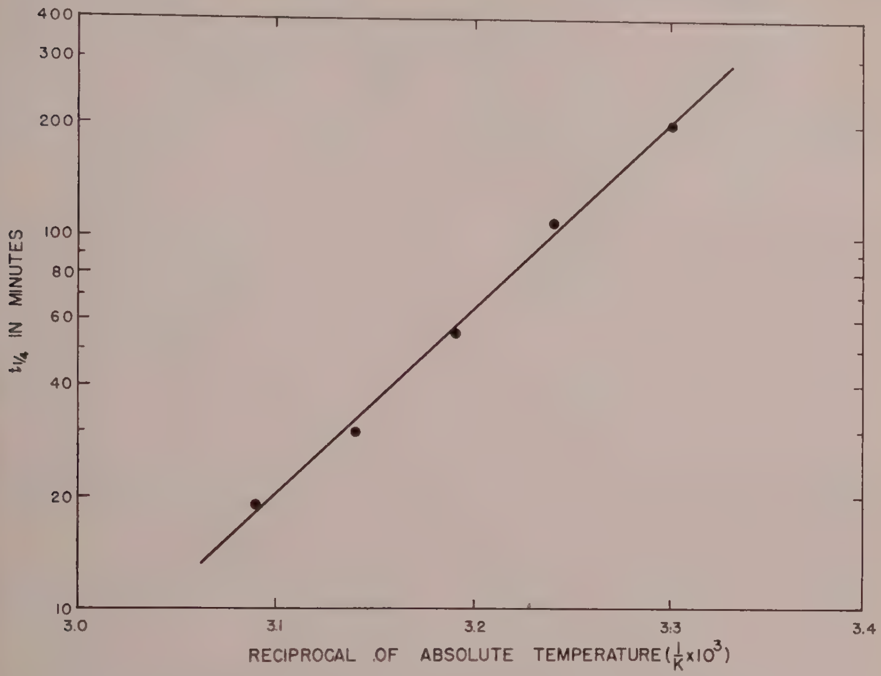

FIGURE 1. Relation between logarithm of the T25 and reciprocal of the absolute temperature for 10-10-100 Bordeaux mixture.

be detected. These samples did not vary greatly in chemical composition or physical properties. However, Bordeaux mixtures made with commercial copper sulfate gave consistently better results than those prepared with the analytical reagent chemical. Crystals obtained from the first and second crystallizations in the purification of copper sulfate gave values of 64 and 76 minutes for t_i compared to 23 minutes for the standard so it was suspected that sphaerocrystal growth was being retarded by impurities which were gradually eliminated during purification. This was confirmed by the finding that the addition of mother liquor from the purification process gave Bordeaux mixtures with unusually high stability.

TABLE III

EFFECT OF SEEDING WITH SPHAEROCRYSTALS ON THE STABILITY OF 10-10-100 BORDEAUX MIXTURE

Source of sphaerocrystals	Amount per 200 ml. of Bordeaux	T25 at 45° C. (minutes)
None	—	29
Unrinsed flask	—	18
48-hour Bordeaux	5 ml.	15
48-hour Bordeaux	10 ml.	14
24-hour Bordeaux	25 ml.	14

TABLE IV

EFFECT OF INORGANIC SALTS ON CRYSTALLITE GROWTH IN 10-10-100 BORDEAUX MIXTURE

Compound No.	Material added*	t_i (minutes)	T25 (minutes)
1	$MgCO_3$	22	26
2	MgO	23	29
3	$K_2H_2Sb_2O_7$	27	30
4	Cu_2Cl_2	25	32
5	Na_2SeO_4	26	32
6	$MnSO_4$	28	34
7	Na_2MoO_4	29	34
8	$NiSO_4$	29	36
9	K_3PO_4	30	37
10	Na_4SiO_4	33	43
11	$FeSO_4$	35	44
12	$NaAsO_2$	40	46
13	$ZnSO_4$	64	72
14	Na_2HAsO_4	—	116

* Tested at a concentration of 8 oz. of the anhydrous salt per 100 gallons of water except for $MnSO_4$ which was tested at a concentration of 1.6 oz. per 100 gallons.

In view of these observations it appeared likely that some inorganic compounds might serve to retard crystallite growth. A number of them were evaluated in 10-10-100 Bordeaux mixture at 45° C. by adding them at a rate of 8 oz. per 100 gallons of water. $MnSO_4$ was so effective that it had to be evaluated at a lower concentration. T25 values varied from 26 minutes for $MgCO_3$ to 116 minutes for Na_2HAsO_4 (Table IV), for the 13 other compounds tested. Mixtures containing manganese sulfate were highly stable at 8 oz. per 100 gallons, but at 1.6 oz. per 100 gallons the T25 was only 34 minutes. Various effects were found when mixtures were used (Table V). A formulation containing 8 oz. ferrous sulfate and 1.6 oz. manganese sulfate per 100 gallons was not appreciably more stable than a mixture containing only the iron salt. However, this was not the case for a preparation containing both zinc and arsenate. The T25 values obtained for these ions when used at 8 oz. per 100 gallons were 72 and 116 minutes

TABLE V

EFFECTS OF MIXTURES OF INORGANIC SALTS AND MOTHER LIQUOR FROM THE CRYSTALLIZATION OF COMMERCIAL COPPER SULFATE ON RETARDING SPHAEROCRYSTAL FORMATION IN 10-10-100 BORDEAUX MIXTURE

Adjuvants in oz. per 100 gal.	t_i (minutes)	T25 (minutes)
6* (1.6 oz.)+11 (8 oz.)	35	45
8 oz. each of 3, 5, 7, 9, and 12	70	80
6 (1.6 oz.)+14 (8 oz.)	—	120
13 (4 oz.)+14 (4 oz.)	120	140
Mother liquor (7 fl. oz.)	34	36
Synthetic mother liquor (7 fl. oz.)	24	28

* Compound No. from Table IV.

TABLE VI

Stabilizing Action of Various Organic Adjuvants on 8-6-100 Bordeaux Mixture

Stabilizer	Amount used (oz. per 100 gallons)	T25 (minutes)
None	—	19
Igepon CN-42	3	12
Triton X-155	3	12
Pluronic F68	3	20
Igepon TK-42	3	25
Triton X-100	3	28
Triton X-114	3	29
Marasperse C	2	120
Sucrose	0.25	30
Sucrose	0.5	81
Sucrose	1	230

respectively, while a mixture containing 4 oz. of each gave a T25 of 140 minutes, thus providing evidence of potentiation. On the other hand, a complex mixture containing a total of 40 oz. of arsenite, molybdate, phosphate, selenate, and antimonate ions per 100 gallons had a T25 of only 80 minutes, showing that the requirements for inhibition of crystallite growth are rather specific. Thus, of the inorganic materials tested, manganous, arsenate, and zinc ions were the most effective, while a combination of zinc with arsenate appeared to act synergistically.

None of the combinations that were tried were as good as the mother liquor obtained during the purification of commercial copper sulfate. Efforts were made to duplicate this material based on a spectrographic analysis provided by the supplier, but the synthetic material was entirely ineffective.

A number of organic adjuvants were evaluated as stabilizers for 8-6-100 Bordeaux mixture. Only T25 values are reported (Table VI) since compositions with less than 10 pounds of copper sulfate per 100 gallons of water became less soluble in sucrose immediately after mixing, even when stabilizers were present. Some surfactants such as Triton X-100 had measurable activity but they were much less efficient than calcium lignosulfonates (Marasperse) and sucrose. This latter compound retarded crystallite growth significantly in amounts as low as 0.5 oz. per 100 gallons, and when the concentration was doubled the stability almost tripled. The decrease in absorbance obtained in the colorimetric measurements on a mixture containing 1 oz. of sucrose per 100 gallons was linear with time. A mixture containing 2 oz. of Marasperse C per 100 gallons behaved similarly for the first 100 minutes, but after this crystallite growth increased rapidly. The same features were observed at lower concentrations of the two adjuvants (Fig. 2).

In order to obtain a direct comparison of the better stabilizers, they

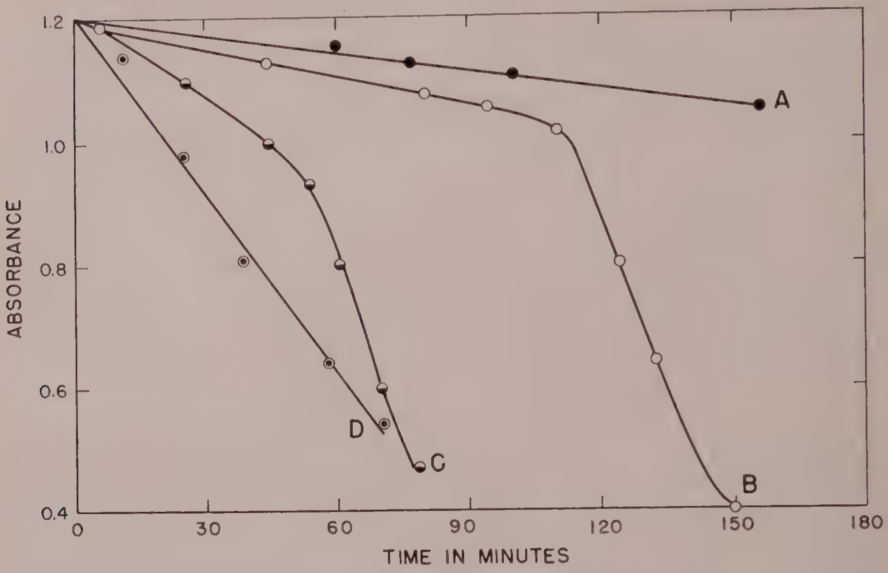

FIGURE 2. Relation between absorbance and time for 8-6-100 Bordeaux mixtures stabilized with (A) 1 oz. sucrose per 100 gallons, (B) 2 oz. Marasperse C per 100 gallons, (C) the same as B but seeded with aged Bordeaux, and (D) 0.25 oz. sucrose per 100 gallons.

were evaluated in 10-10-100 Bordeaux mixtures at a concentration of 1 oz. per 100 gallons (Table VII). In all cases induction periods were obtained.

These experiments showed that the most effective materials were coconut milk solids, sucrose, Maracarb NC, and Marasperse C in that order. The reasons for the outstanding performance of coconut milk solids are unknown. Juice from banana pseudostems also exerted a stabilizing action although this was not measured quantitatively. In any event, it is apparent that some of the organic adjuvants investigated during this program were more effective on a weight basis than any of the inorganic compounds.

The addition of stabilizers ameliorated, but did not entirely cancel out the destabilizing action produced by seeding with sphaerocrystals. Thus,

TABLE VII

RELATIVE EFFECTIVENESS OF ORGANIC STABILIZERS FOR PREVENTING CRYSTALLITE GROWTH IN 10-10-100 BORDEAUX MIXTURE AT 45° C.

Stabilizer	Amount used (oz. per 100 gal.)	t_i (minutes)	T25 (minutes)
None	—	23	29
Casein	8	31	34
Casein hydrolyzate	8	42	70
Marasperse C	1	34	38
Maracarb NC	1	60	60
Sucrose	1	260	270
Coconut milk solids	1	360	380

TABLE VIII

EFFECT OF CONCENTRATION OF BORDEAUX MIXTURES CONTAINING EQUAL AMOUNTS OF COPPER AND LIME ON INDUCTION PERIOD AND T25

Pounds per 100 gallons	t_i (minutes)	T25 (minutes)
8	0	15
12	0	20
16	0	24
20	23	29
24	40	50
32	60	85
40	75	100

the induction period of 10-10-100 Bordeaux mixture prepared with commercial copper sulfate was reduced from 29 minutes to 23 minutes by 5 ml. of seed per 200 ml. of suspension. Similarly, the T25 of 8-6-100 Bordeaux stabilized with 2 oz. of Marasperse C per 100 gallons was reduced from about 120 to 50 minutes by 10 ml. of seed per 200 ml. of Bordeaux in comparison to 19 minutes for the standard. In spray operations the beneficial effects of commercial copper sulfate and adjuvants could be offset, in part, by seeding. The amount of seed used in these experiments was excessive. However, it must be taken into account that the major part of this effect was produced by preparing Bordeaux in an unrinsed container (Table III).

Effect of total solids. Concentrated Bordeaux mixtures were found to be more stable than dilute mixtures when the amounts of copper sulfate and lime were equal. Thus, 20-20-100 Bordeaux had a T25 of 100 minutes compared to 29 minutes for the 10-10-100 mixture. The stability of intermediate compositions decreased progressively as concentration was decreased (Table VIII). The relation between T25 and total solids was approximately linear within this range, but at 10-10-100 Bordeaux mixture a discontinuity occurred (Fig. 3). This composition appears to be critical, since all mixtures with higher total solids form a group with a slope of *ca.* 0.3 lb./min. while mixtures prepared at lower concentrations form a separate group with a slope of 0.6 lb./min.

This sharp division was also reflected in the induction periods. All mixtures containing 20 lb. or more of total solids per 100 gallons of water required at least 20 minutes before changes in their solubilities in solutions of aqueous sucrose could be detected. On the other hand, mixtures containing less than 20 lb. per 100 gallons showed evidence of rapid crystallite growth immediately after mixing (Fig. 4). This was the case for 8-6-100 Bordeaux as well as the 8-8-100 mixture and lower concentrations.

The enhanced stability of Bordeaux prepared at high concentrations was not retained after dilution. A 20-20-100 mixture made at 45° C. and aged 5 minutes before diluting it with an equal volume of water had an induction period of 16 minutes as measured from the time of dilution com-

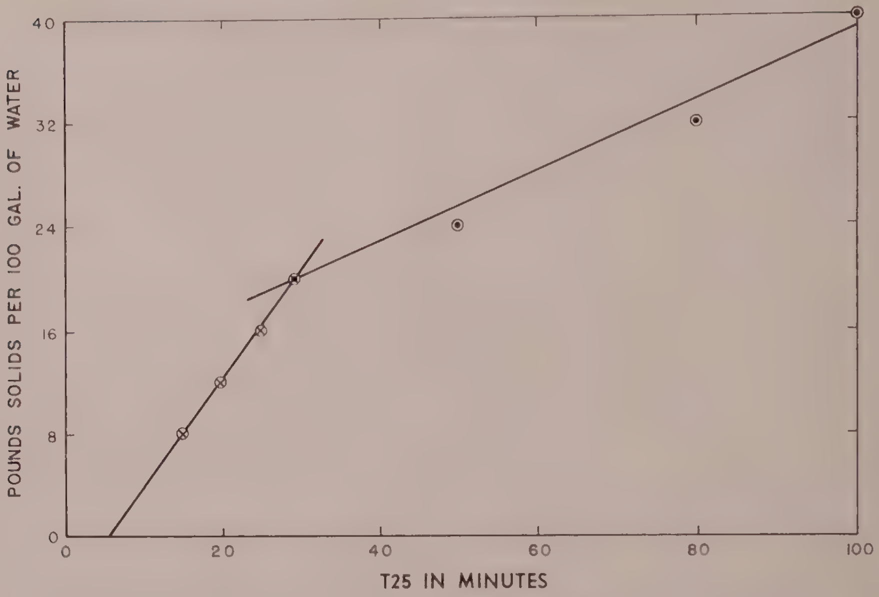

FIGURE 3. Relation between the concentration of solids and T25 of Bordeaux mixtures showing change in slope when the induction period vanishes.

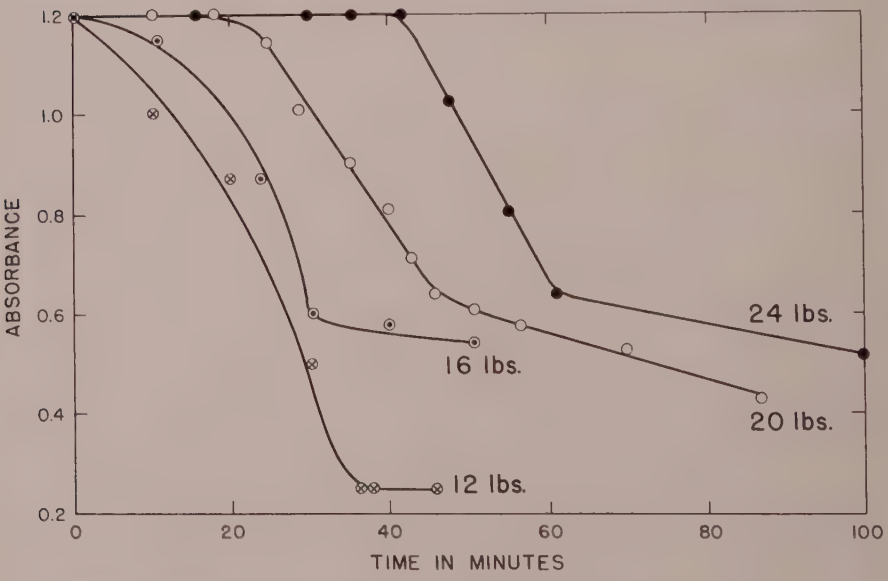

FIGURE 4. Effects of concentration on the stability of Bordeaux mixtures containing the indicated number of pounds per 100 gallons of water of total solids consisting of equal amounts of copper sulfate and lime.

pared to 23 minutes for the standard 10-10-100 formulation. However, 20-20-100 Bordeaux held at 45° C. for 30 minutes before dilution had an induction period (measured from the time of dilution) of 10 minutes which indicates that a temporary stabilizing action was obtained at the higher concentration since 10-10-100 Bordeaux would have formed sphaerocrystals by that time.

TABLE IX

EFFECT OF REPLACEMENT OF SULFATE BY ACETATE OR CHLORIDE ON THE STABILITY OF 10-10-100 BORDEAUX MIXTURE AT 45° C.

Per cent sulfate replaced	With acetate		With chloride	
	t_i (min.)	T25 (min.)	t_i (min.)	T25 (min.)
0	23	29	23	29
25	0	19	0	27
50	0	20	0	29
63	0	25	—	—
75	0	45	0	43
81	0	4	0	—
100	21	43	0	14

The curves relating absorbance (Fig. 4) to time after mixing are of particular interest since they indicate that the properties of the crystallites that were formed varied with initial concentration. Thus, the absorbance obtained on 10-10-100 Bordeaux decreased rapidly to about 0.6 and then fell off more slowly, while the absorbance of the 6-6-100 mixture (corrected to equivalent concentration) decreased to about 0.25 and thereafter remained constant. This could have been caused by larger particle size in the 6-6-100 mixture or the presence of residual hydrogel in the more concentrated Bordeaux.

Effect of sulfate. Significant differences in the results of the chelation test were obtained when part or all of the cupric sulfate was replaced by equimolar amounts of cupric chloride or cupric acetate (Table IX). Preparations containing all copper chloride or mixtures of copper sulfate with chloride or acetate did not have induction periods. By contrast, a 10-10-100 mixture prepared with 100 per cent cupric acetate had a well defined induction period. The shapes of the curves obtained on these mixtures varied considerably (Fig. 5). Thus, when 75 per cent of the sulfate was replaced by acetate or chloride, crystallite growth was very slow and appeared to take place in at least two distinct steps. These mixtures were the most stable in this series as measured by T25. When more than 75 per cent of the sulfate was replaced, a sharp reduction in stability took place. With all of it replaced, the mixture containing acetate became more stable and the mixture containing chloride much less stable. The fact that the greatest

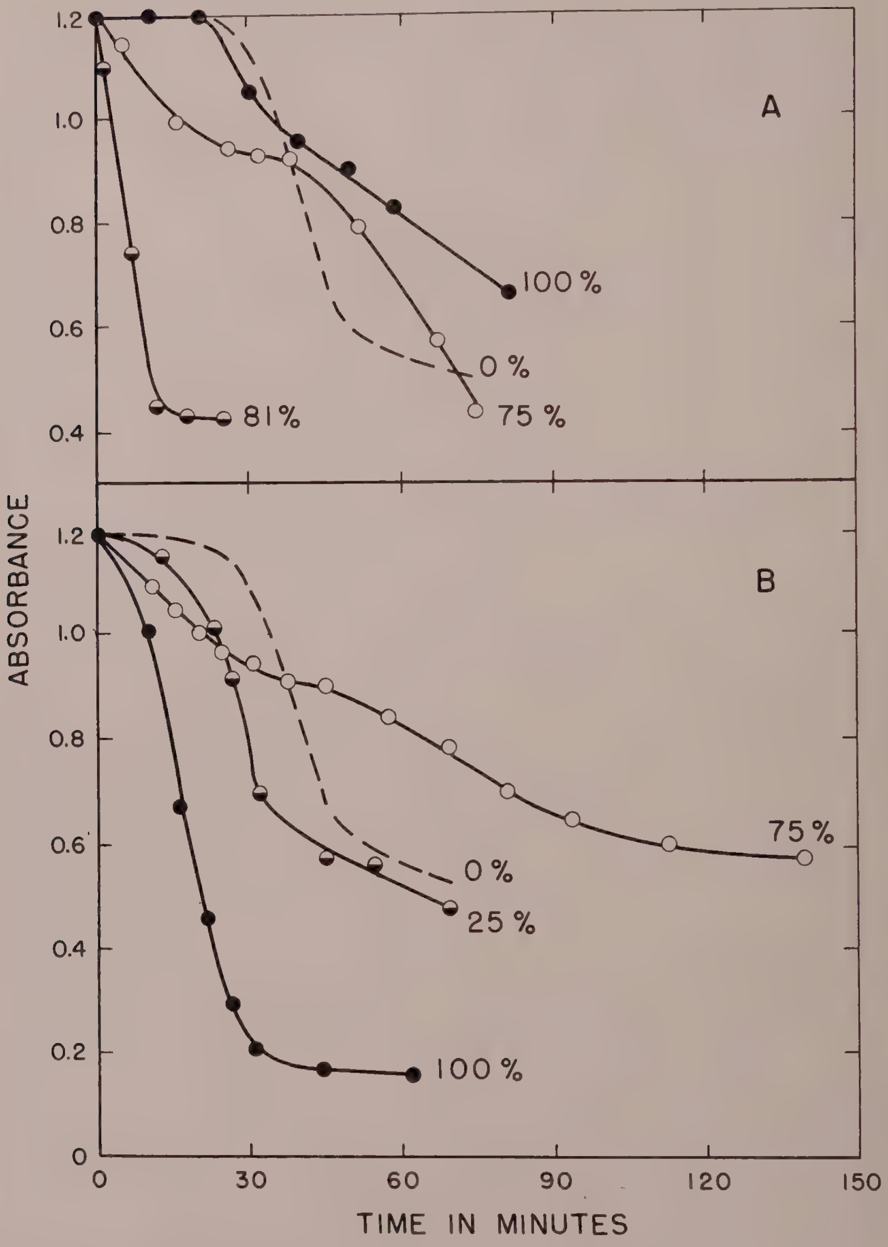

FIGURE 5. Effect of replacement of various percentages of sulfate by acetate (A), and chloride (B) on the stability of Bordeaux mixtures.

changes occurred when about 75 per cent of the sulfate was replaced is significant, since collateral investigations using X-ray diffraction and chemical methods of analysis show that one mole of sulfate per four moles of copper is contained in the crystal lattices of many Bordeaux mixtures. Thus, mixtures containing less than one mole of sulfate would be expected to form different types of compounds.

TABLE X

EFFECTS OF CHANGES IN LIME CONTENT ON T25 VALUES FOR BORDEAUX MIXTURES CONTAINING 10 LB. OF CUPRIC SULFATE PENTAHYDRATE PER 100 GALLONS OF WATER

Lb. of lime per 100 gallons	T25 (minutes)
10	29
8	29
6	40
5.6	50
5.2	60
5.0	80
4.8	100
4.4	350
4.0	*ca.* 5,000
3.0	750

Effect of copper-lime ratio. The most pronounced change in the physical characteristics of Bordeaux mixture containing 10 lb. of copper sulfate pentahydrate per 100 gallons of water occurred on reducing the amount of lime. An increase in stability was observed which was not very great at first since 10-5-100 Bordeaux mixture had a T25 value of only 80 minutes in comparison to about 30 minutes for the 10-10-100 mixture. However, when the amount of lime was reduced to less than 50 per cent of the amount of copper sulfate, stability increased enormously and reached a maximum of about 5000 minutes for the 10-4-100 mixture (Table X). When the amount of lime was decreased to 3 lb. per 100 gallons the T25 dropped to about 750 minutes. In the region of 10-5-100 Bordeaux mixture sphaerocrystals were no longer observed, indicating that this is a critical composition. In terms of stoichiometry it corresponds to 7 moles of calcium hydroxide per 4 moles of cupric sulfate.

The chelation curves obtained when reduced amounts of lime were used differed considerably from those typical of the 10-10-100 mixture. Absorbance values on 10-4-100 Bordeaux remained constant for a few hours and then decreased slowly for a period of about 100 hours. After this the rate of change became more rapid (Fig. 6). The time interval between 5 and 100 hours probably represented a period in which microcrystals were formed. However, they were probably so small that they dissolved in the chelating solution to a considerable extent. After 100 hours at 45° C. the mixture began to convert to CuO and slowly darkened in color. Since CuO

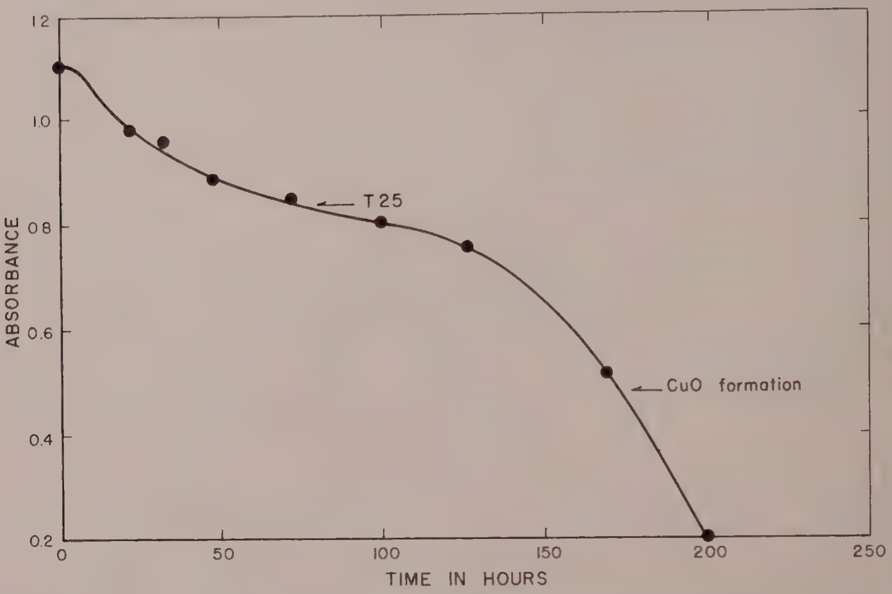

FIGURE 6. Rate of reduction of solubility of 10-4-100 Bordeaux mixture in sucrose at 45° C. The initial lag period of about five hours cannot be seen on this scale.

is insoluble in sucrose the absorbance of the test solutions finally dropped to negligible values.

Small variations in lime content had rather large effects on the results of the chelation test in this range. A 10-3-100 Bordeaux mixture prepared with an equal number of moles of copper and lime had an induction period of about 9 to 10 hours and afterwards the solubility in sucrose began to decrease at a slow rate (Fig. 7). After prolonged standing at room temperature it darkened, probably due to the formation of cupric oxide. This must have represented another critical composition since 10-3.2-100 Bordeaux did not darken under these conditions. On the other hand, the solubility of 10-4.4-100 Bordeaux mixture in sucrose began to diminish immediately after mixing although the rate was rather slow (Fig. 7).

In sedimentation tests on Bordeaux mixtures which had been aged for one month at room temperature, it was found that settling rates paralleled the results obtained by chelation tests to some extent. Thus, mixtures with 5 parts or more of lime settled within a few minutes, while the 10-4-100 mixture had not settled completely 24 hours after mixing. However, the best settling properties were obtained with 3.2 parts of lime (Fig. 8).

GENERAL DISCUSSION

The proclivity of 10-10-100 Bordeaux mixture to form sphaerocrystals is a limiting factor in its effectiveness when the suspension is held in the

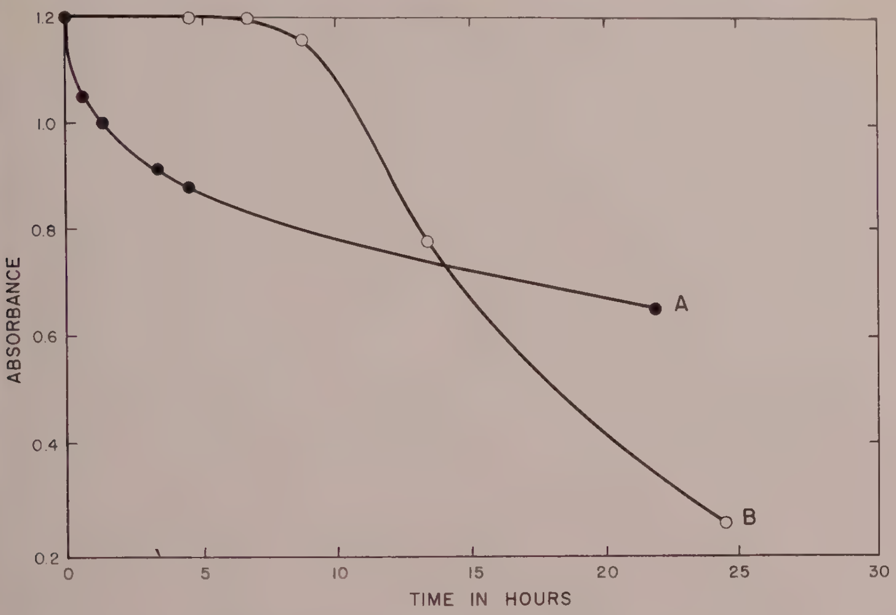

FIGURE 7. Crystallite growth in (A) 10-4.4-100, and (B) 10-3-100 Bordeaux mixtures as measured by decrease in absorbance.

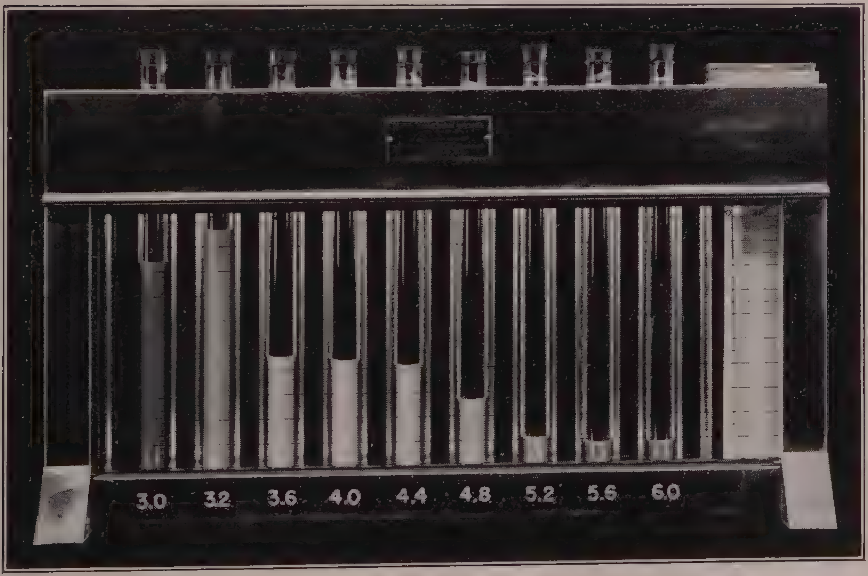

FIGURE 8. Settling during 24 hours of one-month-old Bordeaux mixtures containing various amounts of lime (lb.) in formulations based on 10 lb. copper sulfate pentahydrate per 100 gallons of water.

spray tank for appreciable periods before application. This tendency is increased by high temperatures, low concentrations, and seeding, and retarded by the presence of inorganic and organic stabilizers. Among the inorganic stabilizers manganese, arsenate, and zinc are most effective, while mixtures of zinc and arsenate act synergistically. It is possible that the latter two materials could be used to obtain highly stable formulations which would have both fungicidal and insecticidal properties, and at the same time alleviate copper and zinc nutritional deficiencies. The most effective organic stabilizers tested are coconut milk solids, sucrose, and lignosulfonates, in that order.

However, the most effective way to retard deterioration is by reducing the amount of lime to 40 per cent, or less, of the amount of copper sulfate. Mixtures such as these change very slowly, or, when they do, form very small crystallites which adhere to foliage as well as Bordeaux hydrogel. Some of these mixtures have outstanding settling characteristics even after prolonged aging. There is some tendency for them to form cupric oxide but this conversion is very slow under laboratory conditions. Thus, the formation of cupric oxide was not observed on banana plants which were sprayed with 10-4-100 Bordeaux, subjected to 6 inches of rain, and then placed in a greenhouse for two months. In practice, spray deposits which are stable from one to three weeks would be satisfactory since control of fungus diseases would be lost through leaf expansion and weathering if a longer spray cycle were used. The greatest drawback to the use of these mixtures is the possibility of obtaining phytotoxic effects on crops that are sensitive to copper. This has not been observed on banana plants in the greenhouse but might occur in the field.

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EQUIPMENT FOR PRODUCING SIMULATED RAIN FOR MEASURING THE TENACITY OF SPRAY DEPOSITS TO FOLIAGE

H. P. Burchfield and Alvaro Goenaga

SUMMARY

Simulated rain at a uniform rate and drop size was created by placing a large number of capillary tubes in the bottom of a reservoir in which water was maintained at a constant level. Excised banana leaves or potted plants were exposed to the rain by placing them on a turntable located beneath the reservoir. The surface producing the rain was constructed in the form of a trapezoid so that the rate of fall in inches per hour would be independent of the distance from the center of the table. Controls were provided for varying the rainfall rate from 0.2 to 1.5 inches per hour and the temperature of the water from 40° to 95° F. A few examples are given of the use of this equipment for measuring the tenacities of inorganic and organic fungicides.

INTRODUCTION

A number of methods have been described for measuring the tenacity of spray deposits (7, 8, 9). A procedure developed by Schuldt (8) in which simulated rain is produced by "Teejet" nozzles has been useful in these laboratories for comparing formulations of organic fungicides. However, the rainfall rate of 45 inches per hour is much higher than usually found in nature,[1] while the direction, droplet size, and velocity of impact are arbitrary. In connection with a general program on the relationship between the chemical composition and physical properties of Bordeaux mixtures (1, 2, 3), it was necessary to study the tenacity of spray residues under conditions that would approximate more closely those found in field experiments.[2] To accomplish this an overhead water reservoir equipped with capillary tubes was constructed which supplied simulated rainfall at controlled rates to excised banana leaves or potted plants mounted on a turntable. This equipment is described together with a few examples of its usefulness for evaluating the tenacity of fungicide spray deposits.

MATERIALS AND METHODS

Description of equipment. Simulated rain was applied to excised banana leaves or potted plants at a controlled rate from an overhead reservoir equipped with capillary tubes. The plant materials were mounted on a

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[1] The world's record intensity is variously estimated at 0.65 to 1.03 inches per minute (4, p. 187).

[2] Equipment for measuring the retention of Bordeaux on banana leaves has also been developed by G. B. Savitsky, formerly of the United Fruit Co.

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turntable so they could be moved under the rain surface at constant angular velocity (Fig. 1). The capillary tubes were arranged in the form of a trapezoid so the net rainfall rate would be independent of the positions of the plants when the turntable was in motion. The table was constructed of four semicircles of $\frac{3}{8}$-inch waterproofed plywood, 4 feet in radius, which were glued together to form a disc 8 feet in diameter and $\frac{3}{4}$ inch thick. Four galvanized iron supports spaced equal distances apart were provided for holding banana leaves. The supports were 34 inches long × 14 inches wide $\times\frac{1}{8}$ inch deep and were mounted at angles of 45° so that the projections of their outer edges were chords on the circumference of the table. The turntable was also equipped with 52 1-inch$\times\frac{1}{4}$-inch upright pins for holding potted plants. These were located one foot apart in three concentric circles. The outer circle was 5 inches from the edge of the table and the inner cir-

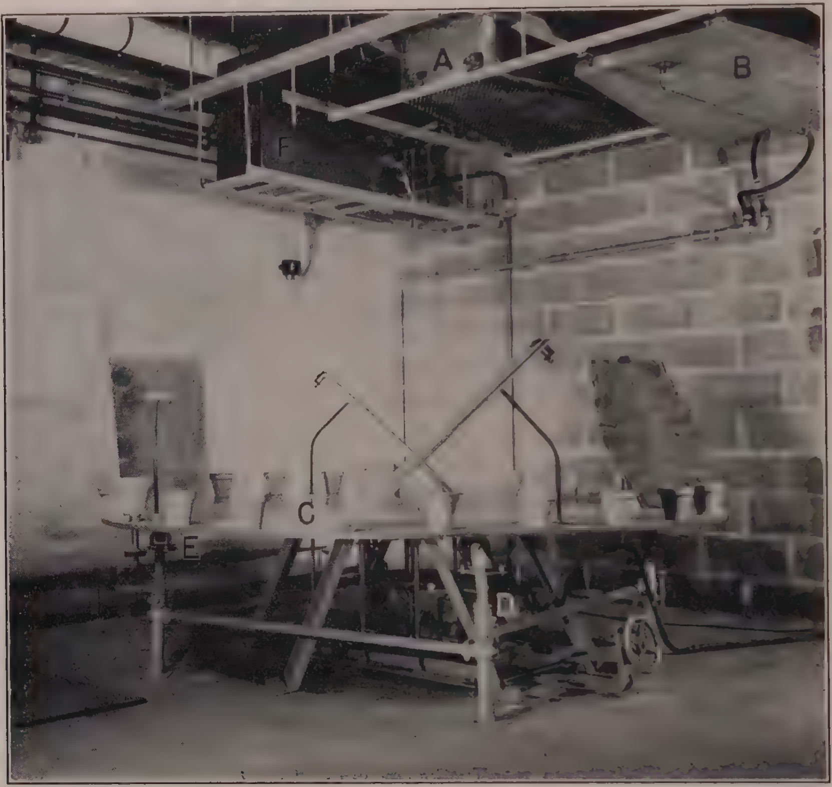

FIGURE 1. Equipment for simulated rainfall. (A) Water reservoir with capillaries. (B) Pan mounted on tracks for intercepting rain. (C) Rotating turntable. (D) Motor and Boston gear. (E) Castor for supporting table. (F) Heat exchanger. The compressor for cooling water is not shown in this photograph.

cles were spaced one foot apart. The turntable was driven at a speed of 10 r.p.m. by a $\frac{3}{4}$ h.p. motor equipped with a Boston gear with a ratio of 15:1. A Warner clutch was installed so that the motor could be started at low speed to avoid throwing off potted plants. The table was supported in the center by the gear axle and in each quadrant, 9 inches from the edge, by a 4-inch rubber rimmed castor mounted on a $1\frac{7}{8}$-inch pipe. The pipes were attached to the floor with flanges and were provided with sleeved unions so that their heights could be adjusted to exactly 2 feet to allow for uniform support of the table.

The water reservoir was constructed of No. 14 gauge stainless steel in the form of a trapezoid with a length of 47 inches and widths of 21 and 41 inches at the ends. It was 10 inches deep and had outlets leading to the drain at 2, 4, 6 and 8 inches from the bottom. Each outlet contained a hand-operated valve and a float valve to maintain the water at the desired level. The inlet valve was located 8 inches above the bottom of the reservoir. The water was passed through a BR10 $\frac{3}{4}$ Fulflo filter (supplied by the Commercial Filter Co., Melrose, Mass.) to remove sediment. It was warmed by a steam hot-water heater capable of raising the temperature of 120 gallons of water per hour from room temperature to 95° F. or was chilled by a cooler capable of reducing the temperature of 120 gallons of water per hour from room temperature to 40° F. The temperature was regulated by a Honeywell Electric Control System. The reservoir was covered to keep out dust.

Water was delivered in uniform sized drops (about 2 to 3 mm.) from capillary tubes 3 cm. long and 0.5 mm. inside diameter, the lower edges of which were beveled down to faces of 2 mm. in diameter. They were fitted in rubber stoppers (No. 00) which in turn were cut down to fit $\frac{1}{2}$-inch holes in the bottom of the reservoir. Prior to installation the capillaries were treated with Desicote (Beckman Instruments Co.) to render them water repellent to prevent the formation of large drops. The capillaries were placed 1 inch apart in 45 rows starting 8 inches beyond the outside rim of the turntable. The row at the edge of the turntable contained 37 capillaries and the innermost row 7. Thus the working rain surface was a trapezoid (with sides coinciding with radii of the turntable) which covered about 40 degrees of arc or one-ninth of the circle formed by the turntable. The reservoir was mounted 6 feet above the surface of the table with the base extending about 9 inches beyond the outer edge. A rain catcher slightly larger than the reservoir and about 2 inches deep was mounted on a track 2 inches below the rain surface so that the droplets could be intercepted by pushing it under the capillaries. The catcher was equipped with a flexible rubber hose leading to the drain. All of the piping beyond the water filter, including the coils in the heater and chiller, was made of stainless steel. The chiller, heater, rain reservoir and temperature control system were supplied and installed by York Distributors Inc., Long Island City, New York.

Test procedures. Leaves of Gros Michel bananas [*Musa sapientum* (L.)] were sprayed with Bordeaux mixtures, exposed to a series of rainfall treatments, and the copper residues determined colorimetrically as described in an accompanying publication (1). Captan and maneb suspensions were applied at 40-lb. pressure to 5-inch potted tomato plants (*Lycopersicon esculentum* Mill. var. Bonny Best) in the standard manner described previously (5, 6). After drying for about 2 hours they were placed on the rotating turntable and exposed to a series of rain treatments in which water at a temperature of 70° F. was delivered at a rate of 1.5 inches per hour. Plants were taken off the table between each treatment so that a series was obtained which was exposed to 0, 0.25, 0.5, 1 and 2 inches of rain.

When the plants had dried they were inoculated with spores of *Alternaria solani* (Ell. & Mart.) Jones & Grout following the procedure developed by McCallan and Wellman (6). The plants were then incubated in an infection chamber at 70° F. and 95 per cent humidity for 24 hours. Lesion counts of disease were made 3 days after inoculation.

Chemicals. The preparation of Bordeaux mixtures has been described elsewhere (2, 3). Rosin soap was supplied by Dr. Melvin Karon of the Standard Fruit and Steamship Co. Captan was a commercial formulation of *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide containing 50 per cent active ingredient. Maneb was a commercial formulation of manganous ethylenebis [dithiocarbamate] containing 70 per cent active ingredient.

APPLICATIONS

Prior to the development of this equipment, prototype apparatus was in use in these laboratories for measuring the tenacity of copper spray deposits (1). It was based on the same general design except that the turntable was 2 feet in radius and only two banana leaves could be mounted on it at a time (Fig. 2). The rainfall rate on the old equipment was 2.5 inches per hour compared to 1.5 inches per hour for the new apparatus, while the peripheral velocity was 9.8 feet per second compared to 4.2 feet per second. The temperature of the rain was not controlled. These differences in operating conditions did not produce major changes in the tenacities of spray deposits containing *ca.* 10 μg. of Cu per cm.^{2} formed from 10-10-100 and 10-4-100 Bordeaux mixtures containing 200 p.p.m. Triton X-114 (Table I). However, the average amount of copper lost on each treatment was about 4 per cent higher on the new equipment, and since the difference was in the same direction in seven out of eight comparisons, it is probable that the erosion produced was slightly more severe.

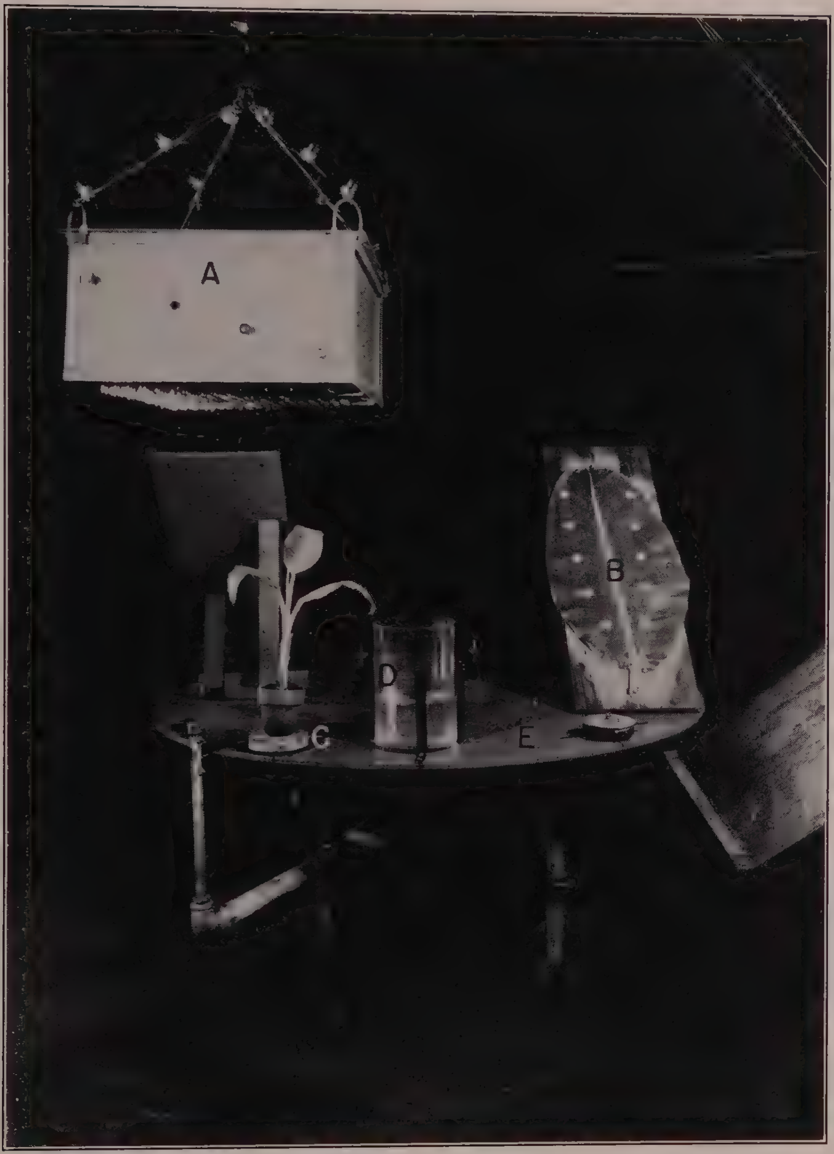

FIGURE 2. Original equipment used in rainfall tests. (A) Rain reservoir showing capillaries and outlets for adjusting water level. (B) Support holding banana leaf at an angle of 45°. (C) Compound turntable holding small potted plant. (D) Rain gauge. (E) Plywood turntable two feet in radius with belt drive adjustable to speeds of 16 and 47 r.p.m.

TABLE I

COMPARISON OF THE TENACITIES OF BORDEAUX SPRAY DEPOSITS ON GROS MICHEL BANANA AS MEASURED ON THE OLD AND NEW RAINFALL EQUIPMENT. BOTH FORMULATIONS CONTAINED 200 P.P.M. TRITON X-114

Inches of rain	Per cent Cu retained in formulation			
	10-10-100		10-4-100	
	Old	New	Old	New
1	84	80	89	87
2	73	64	82	76
4	75	68	78	73
8	68	65	73	74

Small differences in tenacity could be detected if formulations were compared on composite banana leaves (1) so that the average properties of the leaf surfaces were equivalent. Thus in an experiment designed to compare the effects of two levels of urea on Bordeaux mixtures formulated with Triton X-114 or rosin soap, it was found that it improved retention in 19 out of 22 cases when compared with equivalent formulations which did not contain urea (Table II). The average difference in tenacity was only 2.9 per cent which would not be significant if only a few tests were involved. However, the probability that urea has no effect on retention is only about one in 7×10^3 considering this distribution of results.

TABLE II

EFFECT OF UREA ON THE RETENTION OF 10-10-100 BORDEAUX MIXTURES FORMULATED WITH 200 P.P.M. TRITON X-114 OR 600 P.P.M. ROSIN SOAP

Inches of rain	Per cent Cu retained					
	Triton with lb. urea			Rosin soap with lb. urea		
	0	5	10	0	5	10
0.25	93	96	92	20	23	23
0.5	88	94	90	15	19	18
1	85	87	88	10	17	15
2	83	82	86	10	15	13
4	77	78	81	10	13	12
8	70	70	75	—	—	—

The rainfall equipment was also useful for evaluating the tenacity of organic fungicides to tomato foliage using the early blight test described by McCallan and Wellman (6) as an index of performance. Thus when captan and maneb were applied to tomato plants at spray concentrations of 16 to 250 p.p.m., and the plants exposed to 0.25 to 2 inches of rain prior to inoculation, disease control varied in an orderly way with respect to concentration of fungicide and amount of rain even though only one tomato plant was used per treatment (Table III). At low doses of captan, disease control was lost more rapidly than at high doses presumably because the fungicide was present in excess in the latter cases. It is interesting to note that the disease control obtained at 125 and 250 p.p.m. of captan after 2 inches of rain was about the same as found at 16 p.p.m. without rain. This suggests that about 85 to 95 per cent of the fungicide was lost during weathering. Similar results were obtained with maneb although the general level of effectiveness was considerably higher since this material is superior to captan for the control of early blight. Thus at 125 and 250 p.p.m. good control of the disease was obtained at all rainfall treatments, presumably because an excess of fungicide was present initially, and a fraction of it was tenacious enough to withstand the weathering test. However, when the spray concentration was reduced to 16 p.p.m. disease control was lost rapidly with increasing amounts of rain.

TABLE III

ABILITY OF CAPTAN AND MANEB TO CONTROL EARLY BLIGHT OF TOMATO WHEN PLANTS WERE EXPOSED TO VARIOUS AMOUNTS OF SIMULATED RAIN BETWEEN SPRAYING AND INOCULATION

A. Captan

Inches of rain	Per cent disease control at spray concentration in p.p.m.				
	16	32	63	125	250
0	66	86	86	95	99
0.25	49	78	77	90	99
0.5	31	63	67	85	95
1	21	62	71	68	83
2	0	45	63	59	62

B. Maneb

Inches of rain	16	32	63	125	250
0	98	99	99	100	100
0.25	85	96	99	99	100
0.5	75	86	95	99	100
1	51	80	86	97	99
2	40	76	82	97	96

Disease control is not directly proportional to tenacity since the slopes of the dosage-response curves as well as the amount of fungicide per unit leaf area must be taken into consideration. However, it should be noted that 63 p.p.m. of captan without rain gave about the same control as 125 p.p.m. after exposure to 0.5 inch of rain showing that about one-half of the fungicide was lost by this mild treatment. Since 50 to 95 per cent of the spray deposits formed from particulate copper fungicides was lost under similar weathering conditions (1), it seems likely that many inorganic and organic compounds of this class are not utilized as efficiently as Bordeaux mixtures. Thus, it is possible that rainfall tests in conjunction with sensitive chemical methods of analysis can yield information that will be useful in improving the formulation of commercial pesticides.

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SOME FACTORS GOVERNING THE DEPOSITION AND TENACITY OF COPPER FUNGICIDE SPRAYS[1]

H. P. Burchfield and Alvaro Goenaga

SUMMARY

The tenacities of a number of formulations of Bordeaux mixtures and other copper fungicides were measured by determining the amounts of copper retained after exposure to simulated rain. It was found that 10-10-100 Bordeaux mixture had little, if any, ability to adhere to newly emerged leaves of Gros Michel banana unless formulated with surfactants, although the tenacity of the unformulated material was satisfactory on the smooth but nonwaxy surfaces of the cotyledonary leaves of castor beans. Aged 10-10-100 Bordeaux was not retained well on the leaves of banana or castor bean in either the presence or absence of surfactant because of the formation of sphaerocrystals. The growth of these was retarded by sucrose and calcium lignosulfonate, so that aged Bordeaux mixtures containing these materials in addition to a surfactant adhered satisfactorily to banana leaves. On reducing the amount of lime to form 10-4-100 and 10-3.2-100 mixtures, good retention was also obtained even after prolonged aging in the absence of a stabilizer. These latter compositions formed crystallites which adhered to foliage more tenaciously than the sphaerocrystals obtained from the 10-10-100 mixtures, probably because of smaller particle size.

Results obtained on several particulate copper fungicides showed they did not adhere to foliage as well as freshly prepared Bordeaux mixtures. This may have been caused by the fact that freshly prepared Bordeaux is a deformable hydrogel which dries to form films on the foliage surface. These probably fit the contours of the leaves so that the area of contact per unit weight of fungicide is much larger than for wettable powders consisting of discrete particles.

The relation between the amount of rain applied and the logarithm of the tenacity was linear for freshly prepared 10-10-100 Bordeaux mixture deposited on leaves of castor beans. However, a rapid initial loss followed by a slower rate of erosion was obtained on aged Bordeaux mixtures and particulate copper fungicides. It is suggested that a linear relation between the intensity of weathering and the logarithm of the tenacity is characteristic of homogeneous spray deposits, while a curvilinear relation demonstrates that various components of the spray residues differ in their abilities to adhere to foliage surfaces.

INTRODUCTION

The importance of good tenacity to the efficient utilization of foliage fungicides has long been recognized (5, 6, 7, 8, 9, 10). This factor requires particular attention when Bordeaux mixtures are used to control Sigatoka disease of banana since rainfall rates are high in the tropics and the waxy nature of the leaf surfaces makes it difficult to achieve satisfactory spread-

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[1] This work was supported jointly by the United Fruit Co., Boston, Mass., the Phelps Dodge Refining Co., New York, N. Y., and the Standard Fruit and Steamship Co., New Orleans, La.

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ing and coverage. Furthermore Bordeaux mixtures undergo changes on aging which in some cases would be expected to reduce tenacity (2, 3). To obtain information that would be useful in developing improved formulations, the effects of surfactants, aging and composition on the retention of Bordeaux spray deposits were studied. This communication describes the results obtained and compares the tenacities of Bordeaux mixtures to those of some particulate copper fungicides.

MATERIALS AND METHODS

Rainfall apparatus. Tenacity tests on castor beans were carried out by the method of Schuldt (7). Most of the work on banana leaves was done with a prototype of the equipment described by Burchfield and Goenaga (1). This was similar to the newer apparatus except that the radius of the turntable was 2 feet, the peripheral velocity of the table 9.8 feet per second, and the rainfall rate 2.5 inches per hour.

Plant materials. Rhizomes of *Musa sapientum* (L.) var. Gros Michel were planted in soil in four-gallon pails in the greenhouse and leaves were harvested for retention studies when they were about 30 inches long and one foot wide. After repeated harvestings the plants began to retrogress and the leaves became smaller. To obtain more vigorous growth, the pseudostems were cut off about two feet above the level of the soil and the rhizomes replanted in composted garden soil contained in 2-foot × 2-foot × 2-foot boxes. Under these conditions growth was rapid, and the leaves eventually became too large for use on the rain machine. When this occurred the pseudostems were again cut off, the roots trimmed and the rhizomes returned to four-gallon pails, where the leaves soon attained the desired size. By alternating periods of pot binding with relatively free growth and using two groups of plants it was possible to maintain a continuous supply of first leaves[2] of uniform size for residue retention studies. Growth of the plants was satisfactory during the summer months, but was considerably slower during the fall and winter because of lower total light intensity. To alleviate this condition eight 500-watt incandescent and four fluorescent lights were installed in the greenhouse to provide supplementary illumination from 5:00 to 9:00 P.M. from November to March. In addition, 200 ml. of Miracle-Gro[3] (1 tablespoon per gallon of water) were applied to each plant every month to compensate for nutrients lost through harvesting of the leaves and heavy watering of the soil. During the summer months it was necessary to give the greenhouse a coat of shade to prevent excessive wilting. Newly unrolled (first) leaves were used in all experiments except when it was desired to show the effects of leaf age on deposit build-up. Dwarf Cavendish banana (*Musa Cavendishii* Lamb.) was grown under similar conditions in one-foot crocks. Castor bean (*Ricinus communis* L.) seeds obtained from the Baker Castor Oil Co., were sowed, eight to ten per pot, in 6-inch containers and after emergence the seedlings were thinned to four. These were used when the cotyledonary leaves reached full development.

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[2] The first leaf of a banana plant is the youngest fully developed leaf, the second leaf the next youngest, etc.

[3] A commercially available inorganic fertilizer containing the essential nutrients.

The banana leaves used in the retention tests were cut into sections and then reassembled to form composite leaves. This was done to insure that equivalent surfaces would be used when comparing different formulations in a single test.

Spraying of leaves. Two (or four) first leaves about 30 inches long and 12 inches wide were harvested and quartered by cutting them longitudinally down the center and removing part of the midrib, and then cutting them transversely. The quarters were reassembled to form composite leaves by attaching them to $\frac{3}{8}$-inch × 11-inch × 34-inch cedar boards with thumbtacks, taking care not to touch the upper surfaces. When two leaves were used (A and B), the apical right-hand and basal left-hand sides of the first composite leaf were derived from A and the apical left and basal right sides from B. When four leaves were used (1) each of the quarters was selected from a different leaf.

Each composite leaf was sprayed separately by placing it in a horizontal position in the exact center of the floor of a spraying tower consisting of a 4-foot × 4-foot × 8-foot high wooden framework covered with burlap. The sprays were applied with a DeVilbiss gun operated at 40 p.s.i. The gun was mounted on a metal track so that it could be moved back and forth in the direction of the longitudinal axis of the leaf during spraying. Generally a spray time of about 30 seconds was required to obtain an initial deposit of about 10 μg. of Cu per sq. cm. A total volume of 150 ml. was delivered while the gun was moved back and forth along the track about 20 times. This dosage was used throughout these investigations.

Rainfall retention tests. After spraying, the leaves were dried for at least one hour or until it was evident that all of the water had evaporated. Samples were then taken by punching out 2-cm. discs with a cork borer. Eight discs were included in each sample, and five samples taken at each treatment. Sampling was random except that care was taken to see that two discs from each quarter were included in every sample. The leaves then were placed on the rainfall apparatus (1) and subjected to various amounts of rain delivered at a net rate of 2.5 inches per hour. Samples were taken between each treatment as described above.

The copper deposits were removed from the leaf discs by agitating them with 2 ml. of *N* HCl in 50-ml. beakers for 30 seconds. Ten ml. of water were then added to each beaker and the contents transferred to 50-ml. volumetric flasks. Next the leaf sections were washed with two 10-ml. portions of water, and the solutions and washings made up to 50 ml. Aliquots which varied in volume from 5 to 20 ml., depending upon the concentration of copper expected, were then pipetted into 50-ml. flasks and made alkaline with 5 ml. of 7.5 *N* ammonium hydroxide. Colors were developed by adding 2.5 ml. of 0.1 per cent sodium diethyldithiocarbamate and the volumes made up to 50 ml. Absorbances were read at 455 mμ with a Bausch and Lomb colorimeter and the amount of copper in each sample interpolated from a calibration curve obtained from solutions containing known amounts of copper sulfate. Results were calculated in terms of μg. of Cu per cm.^{2} of leaf taking into account only the upper (sprayed) surface.

Measurement of deposit build-up. Banana leaves, sprayed as described previously, were attached to a fiber drum about 18 inches in diameter and 28 inches high. The leaves were thumbtacked to the drum with the midrib of the leaf parallel to the floor while the drum was resting on its bottom. The drum was next placed in an upright position on a turntable 18 inches in diameter (4) and the motor started. Spraying was begun with a DeVilbiss gun while the leaf was shielded by being on the far side of the drum and continued for one full revolution. The motor was then stopped and the leaf sampled by punching out eight 2-cm. discs with a cork borer. The motor was restarted and the leaf sprayed during a second revolution of the drum, after which it was resampled. This was continued until the desired number of treatments was obtained. The samples were then analyzed for copper by the method described above.

Spray deposits on castor beans. Castor beans were sprayed with Bordeaux mixture according to the method described by McCallan and Wellman (4) and subjected to artificial rainfall by the procedure described by Schuldt (7). The amount of copper remaining after each treatment was determined as described above.

Chemicals. Methods used for the preparation of Bordeaux mixtures have been described previously (2, 3). Samples of Marasperse C, Triton X-114, Corvus oil, Binderine flour, and Peronex were supplied by Mr. G. L. Poland of the United Fruit Co., rosin soap and urea were supplied by Dr. Melvin Karon of the Standard Fruit and Steamship Co. Pluronic F-68 was obtained from the Wyandotte Chemicals Corp. and Tween 20 from the Atlas Powder Co. The Triton X-114, Pluronic F-68 and Tween 20 are nonionic surface active agents of various types while Marasperse C is predominantly calcium lignosulfonate.

EXPERIMENTAL RESULTS

REPRODUCIBILITY

Spray deposits, on first leaves of Gros Michel banana, formed from 10-10-100 Bordeaux mixture containing Triton X-114 were gradually eroded with increasing amounts of rain (Table I). In eight independent tests an average of 10 per cent of the initial deposit was removed by 0.5 inch of rain while over 30 per cent was lost after 8 inches. These results indicate that it should be possible to detect differences as small as 8.5 per cent between formulations with significance at the 5 per cent level.

Only two inversions in order were obtained in the eight tests in which deposits from 10-10-100 Bordeaux were subjected to five rainfall treatments (Table I). The most serious one occurred in test 4 in which 90 per cent of the deposit was retained after 0.5 inch of rain and 99 per cent was recorded at one inch. Occasional discrepancies such as these probably arose from sampling errors due to nonuniformity of the initial spray deposit. This was the limiting factor in determining accuracy since deposition tended to be uneven under the best conditions developed for spraying the leaves. This error was minimized by including eight discs from various sections of the leaves in each sample, and taking five samples per treatment.

TABLE I

RETENTION OF FRESH 10-10-100 BORDEAUX MIXTURE FORMULATED WITH 200 P.P.M. TRITON X-114 IN DIFFERENT EXPERIMENTS

Inches of rain	Per cent Cu retained in experiment No.								Average
	1	2	3	4	5	6	7	8	
0.5	88	87	82	90	86	99	90	96	90
1	88	85	85	99	82	93	88	93	89
2	84	81	81	90	81	88	85	88	85
4	79	74	74	77	75	83	75	82	77
8	71	69	65	67	64	79	69	65	69

These data indicate that differences in retention as small as 10 per cent can be detected in independent experiments using different leaves. However, it is possible to demonstrate smaller differences in single tests using a large number of replicates (1).

EFFECT OF SPREADERS ON RETENTION AND DEPOSIT BUILD-UP

Freshly prepared 10-10-100 Bordeaux mixture did not adhere well to newly emerged leaves of Gros Michel banana when subjected to artificial rainfall. Thus after 0.5 inch of rain only 10 per cent of an initial deposit of about 10 μg. per cm.^{2} could be accounted for by colorimetric analysis. Better retention was obtained on older leaves, but in no case was it satisfactory. With Dwarf Cavendish banana the initial loss was also very high but about 30 to 40 per cent of the copper was not removed during the early treatments and subsequently adhered very tenaciously. By contrast, spray deposits formed on the cotyledonary leaves of castor beans were eroded very gradually and the relation between inches of rain and the logarithm

TABLE II

EFFECT OF CONCENTRATION OF TRITON X-114 ON RETENTION OF FRESHLY PREPARED 10-10-100 BORDEAUX TO LEAVES OF GROS MICHEL BANANA

Concentration of Triton X-114 (p.p.m.)	Per cent of initial Cu retained after inches of rain			
	0.5	2	4	8
0	10	0	0	0
100	86	73	65	61
200	94	83	76	76
300	97	93	79	74

of the per cent of the initial deposit retained was linear. These differences in tenacity were probably caused by the properties of the leaf surfaces to which the sprays were applied. The leaves of Gros Michel banana were very waxy and the spray droplets did not spread. Instead they collected at the leaf veins where they dried to form small pellets of Bordeaux which were easily flaked off mechanically. Very little material accumulated in the areas between the veins. The leaves of castor bean, on the other hand, were smooth but not waxy so that the spray deposits were distributed uniformly and did not accumulate in pellets. Dwarf Cavendish banana resembled the Gros Michel variety with respect to the properties of its leaf surfaces but spreading was somewhat better since they were less waxy.

Triton X-114 was evaluated as an adjuvant in 10-10-100 Bordeaux mixture since it was known to be a highly effective surfactant. At a concentration of 100 p.p.m. a remarkable improvement in spreading and retention took place and 86 per cent of the deposit was retained after 0.5 inch of rain in contrast to only 10 per cent in the absence of surfactant (Table II). At the higher dosage levels tenacity was still better, but the greatest improvement occurred on the first addition of the Triton. Similar results were obtained with 8-6-100 Bordeaux and other preparations that were tested so it can be concluded that spreaders are essential for good adhesion when Bordeaux sprays are applied to waxy leaf surfaces.

Several other commercially available surfactants were evaluated in 10-10-100 Bordeaux but none were as efficient as the Triton. Rosin soap at 600 p.p.m. caused a slight but measurable improvement and Pluronic F-68 and Tween 20 were intermediate in effectiveness (Table III). However, these materials represent only a small fraction of the commercial surfactants available so it is possible that other highly effective spreaders could be found by continued investigations.

In addition to improving retention, Triton X-114 also increased the rate of deposit build-up. When banana leaves were attached to a rotating drum and sprayed with standard 10-10-100 Bordeaux, deposition was so slow that after 7 revolutions a total of only 1 μg. per cm.^{2} was accumulated.

TABLE III

EFFECT OF VARIOUS SPREADERS ON THE RETENTION OF FRESH 10-10-100 BORDEAUX BY LEAVES OF GROS MICHEL BANANA

Spreader	Per cent Cu retained after inches of rain				
	0.5	1	2	4	8
None	10	0	0	0	0
600 p.p.m. Rosin soap	14	10	8	6	4
200 p.p.m. Pluronic F-68	36	29	26	18	22
200 p.p.m. Tween 20	59	41	33	28	23
200 p.p.m. Triton X-114	82	85	81	74	65

By contrast, a deposit containing 5 μg. of copper per cm.^{2} was built up from a spray mixture containing 200 p.p.m. Triton X-114. Many of the spray droplets from the formulation which did not contain Triton were observed to hit the leaf surface and bounce off like minute rubber balls, but the mixtures containing the surfactant wet the leaves uniformly. Deposit build-up was found to follow the same pattern as retention with respect to the age of the leaf. After 7 revolutions of the drum, the deposits formed by spraying 10-10-100 Bordeaux contained 1, 2, 4 and 6 μg. of copper per cm.^{2} for first, second, third and fourth leaves of Gros Michel banana respectively. When 200 p.p.m. of the Triton were added, these values were 5, 6, 9 and 10 μg. of Cu per cm.^{2} in order of increasing leaf age. Thus it is seen that the incorporation of a surfactant in the formulation improved both retention and deposit build-up; however, the importance of this factor diminished with leaf age or when plants with nonwaxy leaves were sprayed.

EFFECT OF CRYSTALLITE GROWTH ON RETENTION

Previous investigations on Bordeaux mixtures containing 10 pounds of cupric sulfate pentahydrate and from 5 to 10 pounds of calcium hydroxide per 100 gallons of water (10-5-100 to 10-10-100 mixtures) showed that they changed rapidly from gelatinous precipitates to granular sphaerocrystals, and that the rate of conversion could be determined by measuring solubility in aqueous sucrose (2, 3). To investigate the effects of sphaerocrystal growth on tenacity, the cotyledonary leaves of castor beans were used because they are not waxy and the incorporation of surfactants in the mixtures was unnecessary to obtain good spreading. Freshly prepared 10-10-100 Bordeaux mixture, a mixture that was aged until about half of it was no longer readily soluble in aqueous sucrose (30 minutes at 45° C.), and a sample that was aged for 24 hours at room temperature were evaluated for tenacity according to the method described by Schuldt (7). The effects of aging on retention were pronounced (Fig. 1). The spray deposit formed from the freshly prepared mixture was eroded slowly, only 15 per cent of it being removed by the first two inches of rain. By contrast, about

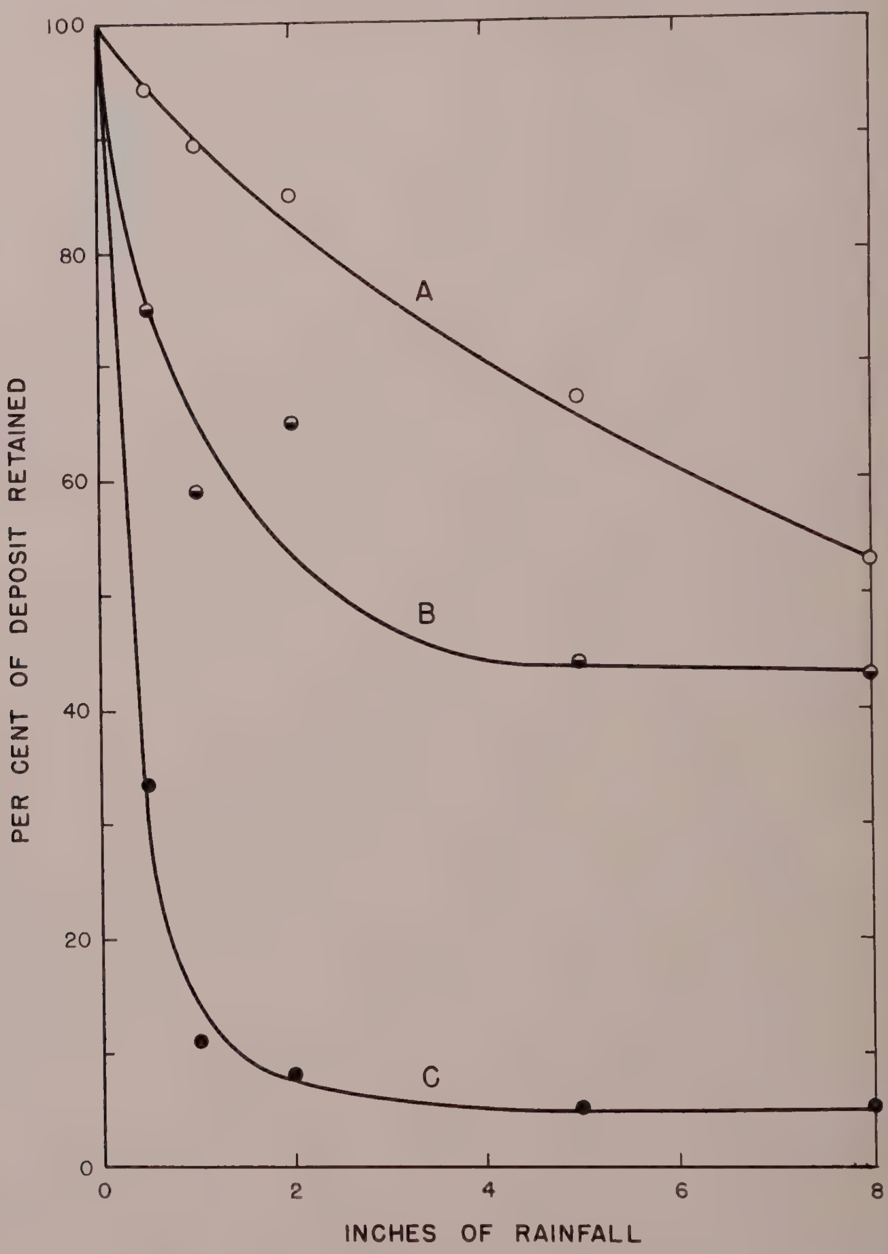

FIGURE 1. Tenacity of 10-10-100 Bordeaux mixtures to the cotyledonary leaves of castor bean when exposed to simulated rainfall. (A) Freshly prepared. (B) Aged to half life (30 minutes at 45° C.). (C) Aged 24 hours.

90 per cent of the aged material was removed by the same treatment, while the mixture that was aged to half life showed intermediate behavior; about 60 per cent of the deposit being removed very rapidly and the remainder adhering tenaciously. These results confirm the usefulness of the sucrose solubility test as a method for evaluating the stability of Bordeaux.

Tests made on first leaves of Gros Michel banana both in the presence and absence of Triton X-114 were even more striking. The aged preparation which did not contain the Triton was completely removed by the first 0.5 inch of rain, while 98 per cent of the deposit formed from an aged preparation containing the Triton was lost under the same conditions, and all of it was removed by one inch of rain. By contrast, only about 3 to 14 per cent of the deposits formed from freshly prepared mixtures formulated with the Triton were lost during the first rainfall treatment while freshly prepared Bordeaux without the Triton failed to adhere.

TABLE IV

RETENTION OF FRESH AND AGED 10-10-100 BORDEAUX MIXTURES FORMULATED WITH 200 P.P.M. TRITON X-114 AND STABILIZERS

Formulation	Per cent Cu retained after inches of rain				
	0.5	1	2	4	8
Fresh (average)	90	89	85	77	69
Aged 24 hours with no stabilizer	0	0	0	0	0
Aged with 480 p.p.m. sucrose	88	84	84	80	61
Aged with 480 p.p.m. Marasperse C	93	86	—	78	66

Thus, it is evident that growth of sphaerocrystals must be retarded and a surface active agent included in the formulation to obtain satisfactory retention of Bordeaux mixtures on banana leaves.

Since the results of sucrose solubility tests had shown that either Marasperse C or sucrose at concentrations of 480 parts per million were able to inhibit sphaerocrystal formation for 24 hours and longer, these materials were evaluated in 10-10-100 Bordeaux mixtures containing 200 p.p.m. Triton X-114. After aging for 24 hours at room temperature both of these formulations adhered to banana leaves as well as freshly prepared Bordeaux (Table IV), thus demonstrating that the deterioration associated with aging can be successfully inhibited by the use of adjuvants.

Earlier studies using the sucrose solubility test (3) had shown that reducing the amount of lime to 40 per cent, or less, of the amount of copper sulfate pentahydrate resulted in mixtures with more desirable physicochemical characteristics. Thus 10-4-100 Bordeaux formed very small crystallites that would be more likely to adhere to foliage satisfactorily than the large sphaerocrystals produced from the 10-10-100 mixture. Retention tests (Table V) showed that freshly prepared 10-4-100 mixture containing

TABLE V

COMPARISON OF RETENTION OF 10-10-100 AND 10-4-100 BORDEAUX MIXTURES FORMULATED WITH 200 P.P.M. TRITON X-114

Formulation	Age	Per cent Cu retained after inches of rain					
		0.5	1	2	4	8	16
10-10-100	Fresh	90	89	85	77	69	49
	24 Hrs.	0	0	0	0	0	0
10-4-100	Fresh	100	93	88	80	61	55
	24 Hrs.	79	79	81	74	63	57

200 p.p.m. of Triton X-114 adhered to banana leaves at least as well as formulations containing larger amounts of lime. After aging the 10-4-100 mixture for 24 hours at room temperature before application, it was found that about 79 per cent of the initial spray deposit was retained on the first application of rainfall, in contrast to a value of about 90 per cent for the fresh 10-10-100 formulation. However, the remaining portion of the deposit proved to be very tenacious. This indicated that aged 10-4-100 mixtures might be slightly inferior in tenacity to freshly prepared mixtures on the initial application of rainfall but were comparable to them afterwards. This reduction in tenacity on the first rainfall treatment was observed in subsequent tests. In an experiment designed to compare fresh, 6-day-old, and 120-day-old 10-4-100 mixtures losses were 7, 15 and 30 per cent respectively after one inch of rain (Table VI). When a 6-day-old mixture was dried and re-wet, about 28 per cent of the deposit was removed by the first inch of rain, thus indicating that some irreversible process took place on drying.

To determine if potential phytotoxic effects arising from the use of mixtures having low lime content could be reduced while still maintaining satisfactory tenacity, a formulation was prepared containing four parts of lime and sufficient calcium carbonate to make the total calcium content equivalent to a 10-10-100 mixture. This adjustment was made on the assumption that free calcium hydroxide would be carbonated rapidly after

TABLE VI

EFFECT OF AGING AND DRYING ON THE RETENTION OF 10-4-100 BORDEAUX MIXTURES CONTAINING 200 P.P.M. TRITON X-114

Treatment	Per cent of Cu retained after inches of rain			
	1	2	4	8
Fresh	93	88	80	61
Aged 6 days	85	85	81	73
Aged 120 days	70	69	65	54
Aged 6 days with $CaCO_3$	61	61	60	54
Dried and re-wet	72	65	59	50

TABLE VII

EFFECT OF CONCENTRATION ON THE RETENTION OF BORDEAUX MIXTURES PREPARED WITH 2.5 PARTS OF COPPER SULFATE PER 1.0 PART OF LIME AFTER AGING FOR 3 WEEKS AT 30° C.

Lb. $CuSO_4 \cdot 5H_2O$ per 100 gallons	Per cent of Cu retained after inches of rain			
	1	2	4	8
4	84	85	79	74
6	87	87	77	70
8	88	83	84	77
10	86	82	86	76

application to foliage. After aging for 6 days, only 61 per cent of the initial deposit (Table VI) was retained after one inch of rain compared to 85 per cent retention for a similar formulation containing no calcium carbonate. Evidently the carbonate impaired adhesion to some extent, but still tenacity was much better than that obtained with aged 10-10-100 Bordeaux.

The concentration at which the Bordeaux was prepared did not have significant effects on tenacity if a 2.5 to 1 ratio was maintained between copper and lime (Table VII). For example, after three weeks aging at 30° C. 86 per cent of a deposit formed from a 10-4-100 formulation was retained after one inch of rain compared to 84 per cent for a 4-1.6-100 mixture, and after 8 inches of rain, retention was 76 and 74 per cent respectively. It can be concluded that mixtures with a ratio of 2.5 parts of cupric sulfate to one part of lime did not deteriorate in a manner analogous to the 10-10-100 preparations regardless of the concentration at which they were prepared.

Bordeaux mixtures prepared with 3.2 parts of lime to 10 parts of copper sulfate also had excellent tenacity on aging. After one week in storage the retention of a 10-3.2-100 mixture was as good as the freshly prepared hydrogel, and probably better than fresh 10-10-100 hydrogel, particularly in the advanced stages of weathering (Table VIII). This composition is of particular interest since it remains hydrated indefinitely in storage (3). As a result it settles more slowly and is resuspended more readily than any other compositions evaluated in these tests.

TABLE VIII

TENACITY OF FRESH 10-3.2-100 BORDEAUX MIXTURE IN COMPARISON WITH A PREPARATION AGED FOR ONE WEEK AT ROOM TEMPERATURE

Material tested	Per cent Cu retained after inches of rain			
	1	2	4	8
Fresh 10-3.2-100	88	88	85	86
Aged 10-3.2-100	93	88	87	82
Fresh 10-10-100 (Average)	89	85	77	69

In addition to the above formulations the only other preparations that were tested extensively were 8-6-100 mixtures containing 200 p.p.m. of Triton X-114. When freshly prepared these adhered as well as fresh 10-10-100 Bordeaux. However, it was shown elsewhere (3) that mixtures containing less than 10 lb. of copper sulfate pentahydrate per 100 gallons of water deteriorated more rapidly and were more difficult to stabilize with adjuvants than 10-10-100 Bordeaux and higher concentrations.

RETENTION OF PARTICULATE FUNGICIDES

During these investigations several tests were made on wettable powder formulations containing copper. These materials were not evaluated as part of an organized program so the data are not always comparable from one test to another. Nevertheless, some interesting trends were noted. It was evident that 10-10-100 Bordeaux was superior in retention to any of the wettable powder formulations tested with the possible exception of a commercial formulation of copper quinolinolate (Table IX). A dried Bordeaux mixture of unknown composition which was submitted for testing failed to adhere better than 10-10-100 sphaerocrystals probably because of large particle size, but, as previously noted (Table VI), dried 10-4-100 Bordeaux gave very good adhesion to banana leaves when resuspended in a solution containing 200 p.p.m. of Triton X-114. A commercial sample of cuprous oxide was tested in a suspension containing 200 p.p.m. Triton and only 53 per cent of the initial deposit was retained after the first 0.5 inch of rain compared to an average tenacity value of 90 per cent for fresh Bordeaux. It is interesting to note that the next half-inch of rain removed only 7 per cent more of the copper and subsequent treatments removed only 1 to 2 per cent per 0.5 inch of rain applied. Evidently almost one-half of the copper was in a form that had little ability to adhere to foliage, but the remainder was considerably more tenacious. It can be seen that formulations of this type need considerable improvement.

TABLE IX

Comparison of the Retention of Particulate Copper Spray Deposits with Bordeaux Hydrogel

Material tested	Per cent Cu retained after inches of rain				
	0.5	1	2	4	8
Fresh 10-10-100 Bordeaux*	90	89	85	77	69
Cuprous oxide	53	46	43	35	—
Dried Bordeaux	4.2	2.8	2.9	2.7	2.5
Copper quinolinolate	—	—	70	61	49

* Average values.

The retention of commercial cuprous oxide to banana leaves was improved by certain additives (Table X). Binderine flour had no effect while Corvus oil gave significantly better retention. Triton X-114 used at a rate of 200 p.p.m. doubled retention at 0.25 inch of rain and tripled it at 2 inches, but no further improvement could be realized by incorporating a sticker in the mixture. It can be concluded that this sample of cuprous oxide was not formulated satisfactorily for use on banana and possibly other plants having waxy leaf surfaces.

TABLE X

EFFECT OF VARIOUS ADDITIVES ON THE RETENTION OF A COMMERCIAL FORMULATION OF Cu_2O TO LEAVES OF GROS MICHEL BANANA

Material added	Per cent Cu retained after inches of rain			
	0.25	0.5	1	2
None	25	21	18	16
Binderine flour	22	22	22	21
Corvus oil	32	30	28	26
Triton X-114	57	53	50	48
Triton X-114 plus National Sticker	55	—	46	43

DISCUSSION

While the use of surface active agents in Bordeaux formulations is probably unnecessary for good tenacity on nonwaxy leaves, it causes a highly significant improvement in the tenacity of spray residues deposited on first leaves of Gros Michel banana because the interfacial tension between the spray droplets and the leaves is reduced which results in good spreading and in the formation of thin films with high contact areas. Without a spreader the droplets collect at the leaf veins, and after drying form small friable pellets which are dislodged mechanically by the first application of rain. Triton X-114 has given outstanding performance as a spreader but probably other materials could be used. Nonionic surfactants which are stable in strongly alkaline media would probably be most successful in this application.

The results obtained with cuprous oxide suggest that many proprietary fungicides may not be formulated suitably for use on banana and other tropical crops with waxy leaves. Most wettable powder formulations probably contain the minimum amount of surfactant required to obtain satisfactory wetting and suspendibility, on the theory that an excess will lead to premature runoff and poor tenacity because of the detergent properties of the adjuvant. This is not the case for Bordeaux-Triton X-114 combinations and it would be worthwhile to determine if this is also true for particulate fungicides. Most organic fungicides are applied at rates of 0.5 to 2 lb. per 100 gallons of water and if they contain about 3 per cent surfactant the spray suspension would contain about 17 to 67 p.p.m. of surface active compound compared to the 100 to 200 p.p.m. of Triton X-114 required to obtain good spreading on banana leaves. Furthermore some of the wetting agents used in these formulations may not be as effective on a weight basis as Triton X-114 for reducing the interfacial tension between banana leaves and water.

In addition to good spreading, prevention of sphaerocrystal growth is also necessary to obtain satisfactory retention of Bordeaux spray deposits. This is true regardless of the nature of the leaf surface to which the mixtures are applied since the sphaerocrystals which form on the deterioration of Bordeaux mixtures containing 5 to 10 lb. of lime per 10 lb. of cupric sulfate pentahydrate are spheres or polyhedra about 5 to 6 μ in diameter which from geometrical considerations would not be expected to adhere well.

This can be illustrated by considering a simplified case in which the only force operating to detach a particle from the under surface of a leaf is gravity. At equilibrium the situation

$$Ap = mg$$

exists where A is the area of contact, ρ is the adhesive force per unit area which binds the particle to the leaf, m is the mass of the particle and g is the acceleration of gravity. If the area of contact is reduced the particle will fall off the leaf and if it is increased it will be bound more firmly since Ap will become larger than mg. If the particle had the shape of an icosahedron of unit volume with 20 equal sides the area of contact would equal the total surface divided by the number of sides or 0.25. If, however, the same mass of material were present as a cube, the area of contact would be unity. If the cube were compressed to form a parallelepiped 0.1 as thick, the area of contact would be 10. Thus it should be possible to increase area of contact, and consequently the adhesive force which binds the particle to the surface by a factor of 40 by changing the shape of the particle from a symmetrical icosahedron (which is analogous to a sphaerocrystal) to a parallelepiped (which is analogous to a film). In addition Bordeaux hydrogel is deformable and on drying should follow the contours of the leaf surfaces more closely than rigid particles. This should also increase tenacity.

Particle size is a second factor which could influence retention since the ratio of the contact area of a single particle to its mass (A/m) becomes larger when the number of particles into which the material is subdivided is increased. For a cube of unit volume and density the ratio A/m would be unity. If this cube were divided into 10^6 sub-units each with cubic structure, the ratios A_i/m_i for the individual particles would be 100. It is assumed that the greater contact area provided by the finely divided material would lead to increased tenacity when spray deposits are subjected to more complicated eroding forces such as wind and rainfall. These speculations are supported by some recent work of Rabe (5) who showed that the retention of sulfur to foliage under artificial rainfall improved with decreasing particle size.

The data obtained on the retention of cuprous oxide to banana leaves suggest that this material was not formulated to provide for optimum performance since almost half of the residue was removed by the first rain treatment. However, the second 0.5 inch of rain removed only 7 per cent of the initial deposit while succeeding treatments removed only about 1 to 2 per cent per 0.5 inch of rain. Thus it is possible that all of the large particles of cuprous oxide and aggregates of small particles were lost in the first treatment while the remainder of the residue consisted of small discrete particles unassociated with their neighbors which resisted erosion more successfully. By contrast freshly prepared Bordeaux deposits on castor bean leaves were eroded slowly in such a way that the relationship between the logarithm of the per cent of copper retained and the inches of rain was linear. A considerable proportion of a spray deposit formed from Bordeaux which had been aged to half life was removed by the first few applications while the remainder was bound more firmly which again suggests a heter-

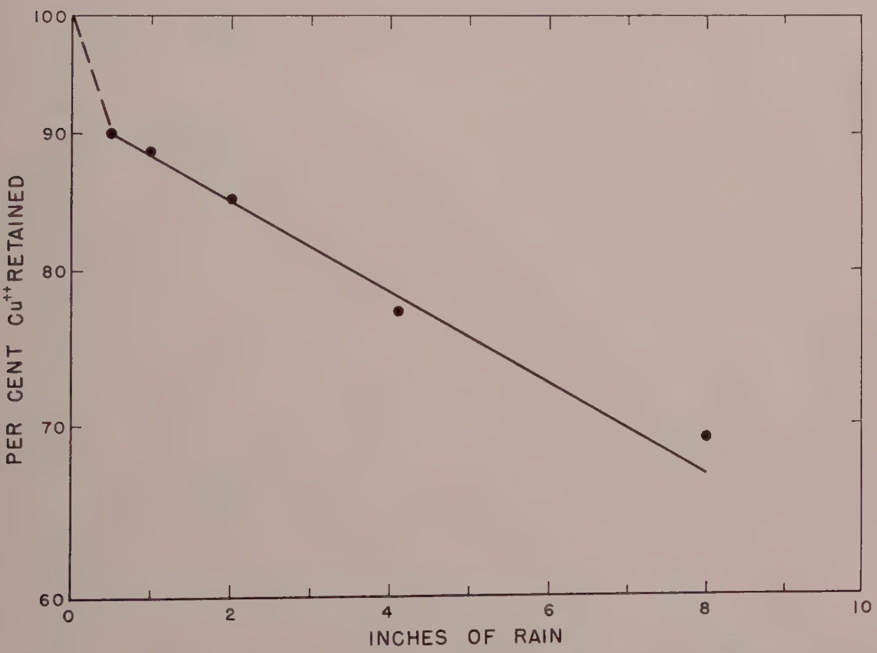

FIGURE 2. Relation between the per cent deposit retained and inches of rain for 10-10-100 Bordeaux+Triton X-114 on banana leaves on a semilogarithmic scale.

ogeneous deposit consisting partly of sphaerocrystals and partly of a film derived from hydrogel. The relationship between the logarithm of the per cent copper retained and inches of rain was also linear for 10-10-100 Bordeaux formulated with Triton X-114 when applied to banana leaves (Fig. 2). However, the curve intercepted the *Y* axis at 90 per cent retention for zero inches of rain which shows that about 10 per cent of the spray deposit was bound very loosely.

These observations suggest that the relation between the logarithm of the spray residue retained and inches of rain is probably linear for homogeneous spray deposits, while heterogeneous deposits will yield curves which are nonlinear or will intercept at less than 100 per cent retention depending upon the ease of removal of the less tenacious component.

The probability that the logarithms of the tenacities of homogeneous spray deposits are directly proportional to inches of rain yields no information on the nature of the surface forces which stabilize spray deposits. The only conclusion this relation suggests is that all components of the deposit adhere equally, and that the amount of fungicide removed with each rainfall treatment is a constant percentage of the amount present at the beginning of each rainfall application.

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EFFECTIVENESS OF VARIOUS FORMULATIONS OF BORDEAUX MIXTURE IN CONTROLLING EARLY AND LATE BLIGHTS OF TOMATO IN GREENHOUSE TESTS[1]

S. E. A. McCallan[2]

SUMMARY

Various formulations of Bordeaux mixture were compared for effectiveness in the control of early and late blights of tomato in repeated greenhouse tests which included exposure to 1 inch of simulated rain after spraying but prior to inoculation. The potted plants were sprayed with undiluted volumes of each formulation to give deposits of 0.32 and 1.60 mg. copper per 100 cm.^{2} of leaf surface. Since the dosage-response curves are exceedingly flat, comparisons were made on the percentage of infection at the two dosages, rather than at an unattainable constant ED level.

Freshly prepared and 24-hour aged 10-10-100 formulations without a rain test did not differ significantly in the control of both diseases. However, following the rain test the aged 10-10-100 formulation gave markedly lower control. An aged 10-4-100 formulation maintained a control equivalent to the fresh preparation or to the fresh 10-10-100 formulation both before and after the rain tests.

In a series of comparative tests, the 10-3.2-100 formulation was more effective than 10-4-100 when applied fresh or aged both before and after exposure to rainfall.

The requisite properties of 10-10-100 Bordeaux as a foliage protectant were prolonged for at least 3 weeks at 30° C. by addition of 0.025 per cent Triton X-114 and 2 per cent Maracarb NC. This preparation controlled both diseases, before and after rain tests, equivalent to that of the freshly prepared 10-10-100 formulation with or without these additives.

While there is a tendency for all the Bordeaux formulations when applied at the higher level of deposit to give a very mild form of necrotic flecking on the tomato leaves, there was no evidence that the 10-3.2-100 or 10-4-100 formulations caused any more flecking than the standard 10-10-100 formulation.

INTRODUCTION

Bordeaux mixture once "the universal fungicide" is still of prime importance in the control of certain diseases. In particular it is used against the Sigatoka disease of bananas (*Musa* sp.) caused by *Mycosphaerella musicola* Leach. Some 60,000,000 or more pounds of copper sulfate are being used annually in Central America alone to control this disease. These control operations are on a large scale using elaborate stationary equipment, and due to the occurrence of rain storms, it is sometimes necessary

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[1] This work was supported jointly by the United Fruit Co., Boston, Mass., the Phelps Dodge Refining Co., New York, N. Y., and the Standard Fruit and Steamship Co., New Orleans, La.

[2] The author is indebted to Miss Doris Long for technical assistance in performing the various tests.

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to suspend spraying operations and hold large quantities of Bordeaux mixture which have been prepared. Thus it is important to obtain information on the stability of various formulations and their subsequent retentiveness and effectiveness in controlling the disease.

Burchfield *et al.* (3, 4) have recently studied the physical chemistry of Bordeaux mixture and noted the superior qualities of certain formulations. This has been confirmed by retention studies on banana and other leaves (2) based on copper analyses. Thus while standard 10-10-100 formulation on aging forms sphaerocrystals with no ability to adhere to leaf surfaces subjected to rain tests, the 10-4-100 formulation forms microcrystals which adhere well. The 10-3.2-100 formulation remains hydrated indefinitely and possesses outstanding settling characteristics. The desirability of the final step of comparing these formulations by direct disease control studies immediately becomes apparent. Despite the successful efforts of Calpouzos (5) in germinating the conidia of *Mycosphaerella musicola*, limited work in the Institute laboratories has not indicated the likelihood of readily obtaining a satisfactory laboratory or greenhouse method of evaluating control of this fungus on banana leaves. It was decided therefore to compare certain of these Bordeaux formulations, freshly prepared and aged, by means of standard greenhouse tests on early and late blights of tomato (7, 8) before and after exposure to artificial rain tests (2).

MATERIALS AND METHODS

The Bordeaux mixture was prepared as described by Burchfield *et al.* (2, 3, 4) to give 10-10-100, 10-4-100, and 10-3.2-100 formulations. These materials were tested immediately after mixing and after standing about 24 hours at room temperature. In addition, freshly made 10-10-100 Bordeaux was compared to the 10-10-100 formulation to which there had been added Triton X-114[3] at the rate of 3 oz. per 100 gal. (0.025 per cent Triton) and 2 per cent Maracarb NC.[4] The latter formulation was tested both freshly made and after standing for 3 weeks at 30° C.

The Bordeaux mixture was sprayed at 40 lb. air pressure on 5-inch potted tomato plants (*Lycopersicon esculentum* Mill.) var. Bonny Best on a turntable in the standard manner described previously (7, 8). Since Bordeaux mixture cannot be diluted without danger of modifying its chemical and physical properties, a dilution series of doses was not applied. Instead different levels were obtained by spraying a specified volume of each formulation. In order to express the amount of spray on a deposition basis extensive calibration tests were necessary. In one series performed at two different times, 5-inch plants were sprayed with 25 and 125 ml. of 10-10-100 Bordeaux, with 3 plants per turntable. Copper analyses were made of the deposition of copper on each of three comparable leaves from each plant. The copper was removed by washing each leaf separately in 100 ml. of 1 per cent hydrochloric acid. A 5-ml. sample was taken, 2 drops of concentrated ammonium hydroxide and 1 ml. of 0.6 per cent sodium diethyldithiocarbamate were added. The intensity of color was determined immediately in a Klett-Summerson photoelectric colorimeter and the amount of copper ascertained from a standard curve. The area of each leaf was measured with a planimeter as previously described (8). The results of this test are shown graphically in Figure 1, and the results from 125 ml. are reduced to one-fifth for direct comparison with 25 ml. It will be noted that the deposition on smaller leaves is proportionally greater than on larger leaves. However, the three leaves chosen, numbers 3, 4 and 5, would be the same as used for disease lesion counts, hence a mean value would be an adequate expression of the deposition. From Figure 1 the mean deposition is 0.484 mg. Cu per 135.4 cm.^{2} or 0.357 mg. Cu per 25 ml. spray suspension per 3 plant turntable. Previous experiments have indicated that with four plants per turntable (the number used in the subsequent disease control tests) the deposition is approximately 10 per cent less. That is 0.32 mg.

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[3] A nonionic alkyl aryl polyether alcohol. Rohm & Haas, Philadelphia, Penna.

[4] A mixture of sodium and calcium lignosulfonates. Marathon Corp., Rothschild, Wisc.

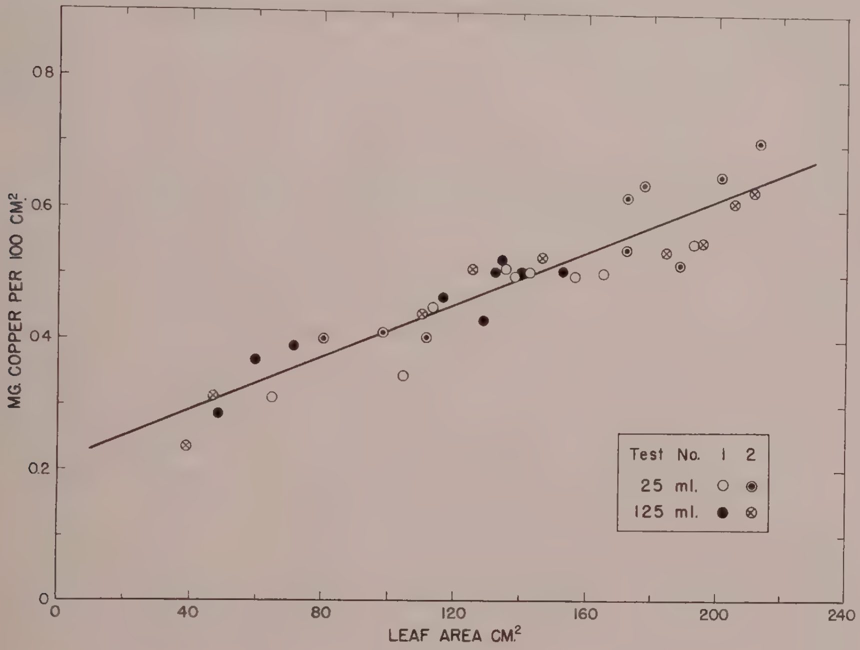

Figure 1. Relation of copper deposition from fresh 10-10-100 Bordeaux mixture to leaf area (both surfaces) of tomato following applications of 25 and 125 ml. For comparison the depositions from 125 ml. were reduced to one-fifth.

Cu per 100 cm.^{2} for a 25-ml. spray and 1.60 mg. Cu for a 125-ml. spray. These two volumes of spray were applied in all tests and about 10 ml. of water used to rinse out the beakers and so ensure that essentially all the volume was sprayed on the plants. Subsequent tests were made in which 10-10-100 and 10-4-100 formulations were compared both freshly made and aged. An analysis of the results showed no significant difference in total copper deposition. Thus in all data subsequently reported the amount of Bordeaux spray is recorded as an initial deposit of 0.32 or 1.60 mg. of Cu per 100 cm.^{2} based on both upper and lower leaf surfaces. The higher deposit is within the ranges obtained under field conditions by Goldsworthy and Green (6). Similar amounts are deposited on bananas under tropical conditions.

After drying for about 2 hours, half the plants were placed on the 8-foot turntable and given 1 inch of artificial rain in 40 minutes. This apparatus has recently been described by Burchfield and Goenaga (1). The temperature of the water was 70° F. In order to prevent excessive washing of the soil in the pots, "sleeves" of thin plastic film were drawn up around the pots and fastened around the lower stem of the plants.

After plants exposed to the rain had dried all plants were inoculated with spores of *Alternaria solani* (Ell. & Mart.) Jones & Grout or swarmspores of *Phytophthora infestans* (Mont.) de Bary, the cause of early and late blight of tomatoes respectively. The procedure was the same as described in 1943 (8) except that newly designed infection chambers were used. The chambers measured approximately 5 feet wide by 5 feet deep by 7 feet high and the inoculated potted plants were placed in flats on specially constructed trucks which are wheeled into the chambers. The chambers were held at 70° F. and 60° F. respectively for the two diseases. Plants were kept in the chambers for 24 hours at a humidity of 95 per cent or more, then returned to the greenhouses. Lesion counts of disease were made, respectively, 3 and 4 days after inoculation. No consistent difference was found in the number of lesions on unsprayed plants exposed to rain and the controls, so the lesion counts for the two groups were combined. The number of lesions per three compound leaves per plant ranged from about 120 to 1200 for the different tests and averaged about 600 for early blight and 800 for late blight. The results were expressed as percentage infection based on the control plants. All variables were replicated on three plants for a given test and the tests were replicated from 3 to 5 times. Some 2000 tomato plants were used in the course of this study.

RESULTS

The slope of the dosage-response curve for Bordeaux mixture in the control of early and late blights of tomato in greenhouse tests is known to be very flat (8). This was again demonstrated in the present study as shown in Figure 2. However, in this study only two doses, differing 5-fold, were applied so that only a small range was obtained in the percentage of control. Also because of the extreme flatness of the slopes, curves essentially without slope or even with negative slopes were sometimes obtained. Due to these limitations it was not possible to obtain without undue extrapolation a given ED value for a series of curves. Instead the percentage infection at the two deposit levels has been used as the measurement of effectiveness. All the data so obtained have been examined by an analysis of variance (10). It was emphasized earlier (9) that the comparison of fungicides should not be based on the internal variability for a given test but rather on the consistency with which the fungicides behave in different tests. Thus in the analysis of variance, discrepance is usually an inadequate source of error, and instead the usually significant interaction of fungicides or treatments with tests is the appropriate error. While this makes it more difficult to demonstrate significant difference, it nevertheless rests on a broader base of performance in different tests.

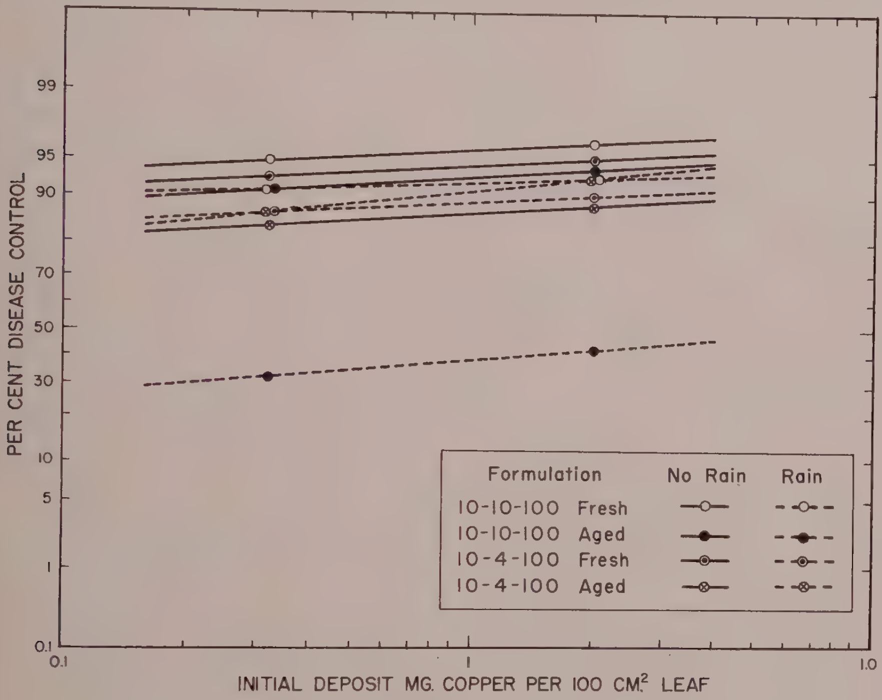

Figure 2. Dosage-response curves for control of tomato early blight by various formulations of Bordeaux mixture, with and without a 1-inch rain test, showing marked flatness of slope and ineffectiveness of aged 10-10-100 formulation following rain treatment. A single test.

TABLE I

Effectiveness of Fresh and Aged 10-10-100 Formulations of Bordeaux Mixture in Controlling Early and Late Blights of Tomato, Based on Means of Three Tests Each

A. Per cent infection

Bordeaux formulation	Initial deposit mg. Cu per 100 cm.^{2}	Early blight		Late blight	
		No rain	1″ Rain	No rain	1″ Rain
10-10-100 Fresh	0.32	9.5	18.6	3.3	4.6
	1.60	7.5	12.6	1.4	2.7
	Mean	8.5	15.6	2.3	3.7
10-10-100 Aged	0.32	12.8	41.4	6.0	25.3
	1.60	7.9	31.0	1.7	8.6
	Mean	10.3	36.2	3.8	17.0
Significant difference between means successive ranks apart		16.1, 20.2, 22.8		18.8, 23.6, 26.6	

B. Estimated ED values, mg. Cu per 100 cm.^{2}

	ED90		ED95	
10-10-100 Fresh	0.22	3.8	0.15	0.26
10-10-100 Aged	0.75	1000	0.42	3.10

The customary least significant difference (L.S.D.) is correctly applicable only to two treatments and larger differences are required as the number of treatments increases. By means of the *Q* table it is possible to extend the L.S.D. to cover multiple treatments by the method of Hartley according to Snedecor (10, p. 253). In the present studies mean values are given for the two deposits and the various replicated tests, and the required difference for significance at the 5 per cent level between these means is given under the heading "Significant differences between means successive ranks apart." Thus for example in Table I the four means for the early blight data are in order of rank: 8.5, 10.3, 15.6, and 36.2. The required differences for significance are for adjacent treatments 16.1, for treatments 2 ranks apart 20.2, and for 3 ranks apart 22.8. Hence it will be seen that the first three treatments do not differ significantly from one another, but that all three are superior to the fourth treatment.

Approximate ED values based on the means for the deposits of 0.32 and 1.60 mg. of copper for all tests are given in the "B" portion of each table for orientation purposes. The actual level for ED values which differ in the tables and for the two diseases was selected so as to be near the middle of the range of reported percentage of infections in order to avoid excessive extrapolation. It will be realized that very large differences between these ED values are necessary for significance.

TABLE II

Effectiveness of Fresh and Aged 10-10-100 and 10-4-100 Formulations of Bordeaux Mixture in Controlling Early and Late Blights of Tomato, Based on Means of Four Tests Each

A. Per cent infection

Bordeaux formulation	Initial deposit mg. Cu per 100 cm.^{2}	Early blight		Late blight	
		No rain	1″ Rain	No rain	1″ Rain
10-10-100 Fresh	0.32	9.7	14.1	1.8	4.5
	1.60	4.7	10.5	1.1	1.7
	Mean	7.2	12.3	1.4	3.1
10-10-100 Aged	0.32	13.2	56.1	9.0	40.8
	1.60	7.9	42.8	3.6	21.7
	Mean	10.6	49.4	6.3	31.3
10-4-100 Fresh	0.32	8.7	14.0	3.0	3.8
	1.60	6.1	9.3	1.4	3.0
	Mean	7.4	11.7	2.2	3.4
10-4-100 Aged	0.32	12.9	12.0	4.8	3.3
	1.60	8.4	11.0	1.8	2.0
	Mean	10.7	11.5	3.3	2.7
Significant difference between means successive ranks apart		4.7, 5.6, 6.2, 6.7, 7.0, 7.2, 7.3		8.3, 10.2, 11.4, 12.3, 12.9, 12.5, 14.0	

B. Estimated ED values, mg. Cu per 100 cm.^{2}

	ED90		ED95	
10-10-100 Fresh	0.30	2.0	0.01	0.27
10-10-100 Aged	0.80	400	0.90	20
10-4-100 Fresh	0.16	1.3	0.10	0.10
10-4-100 Aged	0.80	4.0	0.30	0.10

In Table I the mean results are presented for three tests on fresh and aged 10-10-100 Bordeaux mixture with and without being subjected to the rain test prior to exposure to the two pathogens. It will be seen that without a rain test there is no significant difference between freshly made or aged 10-10-100 Bordeaux in the control of either late or early blight. However, following the rain treatment the aged 10-10-100 Bordeaux lost greatly in effectiveness against early blight. Although there was a reduction in control of late blight, it was not significant at the 5 per cent level.

In a second series, comparisons were made of 10-10-100 and 10-4-100 formulations of Bordeaux mixture both freshly made and aged for 24 hours. The mean results of four tests are given in Table II. Again there is no appreciable difference between fresh and aged Bordeaux of 10-10-100 or 10-4-100 formulation with no rain test for either early or late blight, and the aged 10-10-100 Bordeaux after the rain test failed markedly in the control of both diseases. However, it is significant that the aged 10-4-100 formulation of Bordeaux withstood the rain test and controlled both diseases as well as the freshly prepared 10-10-100 or 10-4-100 formulations.

Comparisons were made of 10-4-100 and 10-3.2-100 formulations of freshly made and aged Bordeaux in a third series consisting of five tests.

TABLE III

EFFECTIVENESS OF FRESH AND AGED 10-4-100 AND 10-3.2-100 FORMULATIONS OF BORDEAUX MIXTURE IN CONTROLLING EARLY AND LATE BLIGHT OF TOMATO, BASED ON MEANS OF FIVE TESTS EACH

A. Per cent infection

Bordeaux formulation	Initial deposit mg. Cu per 100 cm.^{2}	Early blight		Late blight	
		No rain	1″ Rain	No rain	1″ Rain
10-4-100 Fresh	0.32	5.6	9.5	1.8	3.0
	1.60	3.3	6.3	1.0	2.4
	Mean	4.4	7.9	1.4	2.7
10-4-100 Aged	0.32	5.0	8.2	1.2	2.2
	1.60	3.8	5.9	0.8	1.4
	Mean	4.4	7.0	1.0	1.8
10-3.2-100 Fresh	0.32	4.5	7.3	1.5	2.8
	1.60	3.8	6.0	0.8	1.9
	Mean	4.1	6.6	1.2	2.3
10-3.2-100 Aged	0.32	4.5	6.7	1.2	2.2
	1.60	3.3	5.3	0.7	1.5
	Mean	3.9	6.0	0.9	1.8
Significant difference between means successive ranks apart		1.4, 1.7, 1.9, 2.0, 2.1, 2.1, 2.2		0.6, 0.7, 0.8, 0.8, 0.9, 0.9, 0.9	

B. Estimated ED values, mg. Cu per 100 cm.^{2}

	ED95		ED98	
10-4-100 Fresh	0.45	4.0	0.20	50
10-4-100 Aged	0.30	3.5	0.05	0.5
10-3.2-100 Fresh	0.20	5.0	0.12	1.3
10-3.2-100 Aged	0.20	2.5	0.08	8.0

The mean results are shown in Table III. In general it was found that the 10-3.2-100 formulations were significantly superior to the 10-4-100 formulations in the control of early blight both with and without rain tests. While favorable differences for the 10-3.2-100 formulation also hold for the results with late blight they are not significant at the 5 per cent level. The aged preparations of both the 10-4-100 and 10-3.2-100 formulations were at least as effective in disease control as the freshly made preparations and with late blight showed a significant superiority.

Since there may be some question of the practicality of applying Bordeaux mixtures with reduced lime content such as the 10-4-100 or 10-3.2-100, studies were made of the effectiveness of Triton X-114 and Maracarb NC (2, 4) in prolonging the active life of the standard 10-10-100 Bordeaux. Five tests were made of freshly prepared 10-10-100 Bordeaux, the same formulated with 0.025 per cent Triton X-114 and 2 per cent Maracarb NC

TABLE IV

EFFECTIVENESS OF FRESH AND AGED 10-10-100 BORDEAUX MIXTURE FORMULATED WITH TRITON X-114 AND MARACARB NC IN CONTROLLING EARLY AND LATE BLIGHT OF TOMATO, BASED ON FIVE TESTS EACH

A. Per cent infection

Bordeaux formulation	Initial deposit mg. Cu per 100 cm.²	Early blight		Late blight	
		No rain	1″ Rain	No rain	1″ Rain
10-10-100 Fresh	0.32	8.2	9.0	6.8	7.3
	1.60	5.8	10.0	3.6	4.3
	Mean	7.0	9.5	5.2	5.8
10-10-100 Fresh +0.025% Triton X-114 +2.0% Maracarb NC	0.32	11.2	10.2	7.5	7.9
	1.60	4.1	4.6	5.1	4.1
	Mean	7.6	7.4	6.3	6.0
10-10-100 Aged +0.025% Triton X-114 +2.0% Maracarb NC	0.32	8.8	11.8	5.8	10.9
	1.60	6.7	7.1	2.9	4.0
	Mean	7.7	9.4	4.3	7.4
Difference between means		Not significant		Not significant	

B. Estimated ED values, mg. Cu per 100 cm.²

	ED90		ED95	
10-10-100 Fresh	0.13	?	0.75	1.00
10-10-100 Fresh+Triton, Maracarb	0.38	0.33	1.50	1.00
10-10-100 Aged+Triton, Maracarb	0.16	0.55	0.50	1.25

and thirdly the preparation with additives which had been stored for 3 weeks at 30° C. The mean results are reported in Table IV. It will be noted that Triton X-114 and Maracarb NC added to 10-10-100 Bordeaux mixture successfully extended its activity for at least 3 weeks at 30° C., since at the end of this period the control of both early and late blights was equal to that of the freshly made 10-10-100 Bordeaux either with or without these additives. No consistent difference could be shown between the three formulations.

In all five tests with early blight where the 10-10-100 freshly prepared Bordeaux without additives was exposed to rainfall, the plants with lower deposit of 0.32 mg. copper appeared to have fewer infections than those with the higher deposit of 1.60 mg. This reversal did not occur prior to the rain treatment nor with late blight. There is a tendency for all the Bordeaux formulations applied at the higher dose to give a very mild form of injury characterized by small necrotic flecks. Since the series of tests reported in Table IV were performed during midsummer it is possible that these higher temperatures together with those of the early blight infection chamber in association with the softening process of the rain test may have caused additional necrotic flecking which could not be clearly differentiated from the early blight lesions. Thus this reversal could have been brought about by a failure to recognize as disease lesions all those present at the lower dose, or to include as disease lesions various necrotic flecks at the higher dose, or both. A comparison with similar data on fresh 10-10-100 Bordeaux in Tables I and II would indicate the first possibility. If this were the case, then the Bordeaux with the additives possibly would be superior to that without, in the control of early blight following the rain test.

DISCUSSION

These greenhouse tests on the control of early and late blights of tomato have confirmed the physical chemical studies of Burchfield, Schechtman and Magdoff (4) on the stability of various formulations of Bordeaux mixture, and those of Burchfield and Goenaga (2) on retentiveness to banana foliage following rain tests. Thus upon aging the 10-10-100 Bordeaux formulation forms large sphaerocrystals which lack retentiveness when subjected to rain tests and hence are ineffective in the control of early and late blights. On the other hand the 10-4-100 formulation, which on aging forms microcrystals, adheres well to foliage during rain tests and maintains a high degree of plant disease control, as does also the 10-3.2-100 formulation which has been shown to settle very slowly. The addition of the surfactants, Triton X-114 and Maracarb NC, which stabilize 10-10-100 Bordeaux and markedly improve retentiveness also enable this formulation when aged to maintain a disease control equivalent to that of the freshly prepared formulation.

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RELATIVE REACTIVITIES OF 1-FLUORO-2,4-DINITROBENZENE AND 2,4-DICHLORO-6-(*o*-CHLOROANILINO)-*s*-TRIAZINE WITH METABOLITES CONTAINING VARIOUS FUNCTIONAL GROUPS

H. P. Burchfield and Eleanor E. Storrs

SUMMARY

The second order velocity coefficients for the reactions of a number of metabolites and related compounds with the fungicides 1-fluoro-2,4-dinitrobenzene (FDNB) and 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine were determined at 29° C. and pH values of 7.0 or 8.0. With the exceptions of *p*-aminobenzoic acid and nicotinic acid which have pK′ values considerably lower than the pH values of the media, the apparent rate constants were dependent on hydrogen ion activity. This was shown to be the case for compounds containing aliphatic amino, aromatic hydroxyl, and sulfhydryl groups, so intrinsic velocity coefficients were calculated using an equation which contains terms for the dissociation constant of the reactive group and the hydrogen ion activity of the medium. Before and after this correction it was found that the reactivities of the *s*-triazine and FDNB with a group of 7 compounds containing primary amino groups paralleled one another. Evidently the reactivities of the two toxicants respond in the same general manner with respect to changes in the structures of the substrates. However, very large differences in relative reactivity were observed between compounds containing various functional groups. Thus FDNB reacted only 2 to 10 per cent as rapidly as the *s*-triazine with compounds containing amino, phenolic, and heterocyclic nitrogen groups, but combined with glutathione at about the same rate. This relatively high specificity for sulfhydryl groups may ultimately explain why FDNB is a more effective compound than the *s*-triazine for the control of soil borne fungi.

INTRODUCTION

Recent investigations on the rates of reaction of various metabolites with the fungicide 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine (2) showed that second order velocity coefficients could be correlated with the chemical structures and dissociation constants of the substrates. Furthermore it was found that isomers of this compound with the chlorine atom in the benzene ring substituted in the *meta* or *para* positions reacted at slower rates, although their relative reactivities with such dissimilar metabolites as glycine and cysteine were approximately the same (3). Thus it might be inferred that toxicants which function like alkylating agents parallel one another in reactivity, so that while it is possible to synthesize molecules differing considerably in absolute reactivity, it would be unlikely that they would differ significantly in their specificities for different metabobolites. However this supposition does not take into account the fact that the nature of the transition state formed between reacting molecules, and

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thus the energy of activation required to cause them to combine with one another, can differ considerably, even when the mechanisms of the reactions appear to be similar superficially. Furthermore, frequency factors might also be altered by differences in the signs of the charges on reacting ion pairs, while steric effects are believed to be extremely important in reactions with large molecules. Thus, the possibility that toxicants possessing a relatively high degree of specificity will be found, cannot be dismissed.

These present investigations compare the reactivities of 1-fluoro-2,4-dinitrobenzene (FDNB) and an *s*-triazine with a number of metabolites and related compounds. FDNB was chosen for these studies since it is distinctly different from the triazine in the nature of its functional and activating groups, while prior studies by Singh and Peacock (11), and Chapman *et al.* (4, 5) showed that it combines with aromatic amines more rapidly than its chlorine analogue. Furthermore this compound is an effective fungicide (7) and also has been used extensively as a reagent for the analysis of amino acids and proteins (6, 8, 9, 10) so that its reactions with metabolites are of general interest.

MATERIALS AND METHODS

Chemicals. Glutathione, proline, taurine, *p*-aminobenzoic acid, leucine, nicotinic acid, α-alanine and β-alanine were obtained from the Nutritional Biochemicals Corp. Glycine was obtained from the Calco Chemical Corp., ethylamine hydrochloride and γ-aminobutyric acid from the California Foundation for Biochemical Research, while *p*-hydroxybenzoic acid and dichloromethane were supplied by the Eastman Kodak Co. 1-Fluoro-2,4-dinitrobenzene (hereafter referred to as FDNB) was obtained from the Aldrich Chemical Co., pyridine from the Mallinckrodt Chemical Co. and tetraethylene glycol dimethyl ether from the Ansul Chemical Co. The preparation and properties of 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine (hereinafter referred to as the *s*-triazine) have been described elsewhere (2). An 11.7 per cent by volume solution of pyridine in water and $7N$ aqueous sodium hydroxide were used as reagents for analyzing for FDNB and the *s*-triazine (1).

Measurement of reaction rates. Reaction rates were measured at pH 7.0 and pH 8.0 in $M/30$ phosphate buffer containing 1 per cent acetone at 29° C. The reaction mixtures were prepared with deionized water and kept under an atmosphere of nitrogen. The initial concentration of FDNB or triazine was 5 μg. per ml. while 10 to 4,000 molecular equivalents of substrate were used depending upon their reactivities. Ten ml. samples were withdrawn at various times and extracted with 10 ml. of dichloromethane to stop the reaction. Tetraethylene glycol dimethyl ether (0.08 ml.) was added to 5 ml. aliquots of the dichloromethane layers and the solvent removed in a current of air. Six ml. of pyridine reagent were then added, and after 20 minutes the colors were developed with 1 ml. of 7*N* sodium hydroxide. They were measured two minutes after the addition of alkali at 548 mμ for the FDNB and 440 mμ for the *s*-triazine. Second order velocity constants in units of mole liter^{-1}sec.$^{-1}$ were calculated on the assumption that bimolecular reactions took place between the toxicants and the substrates. In all cases the reactions were first order with respect to the rate of disappearance of the toxicants since a large excess of substrate was always present.

MODIFICATIONS IN PROCEDURE

In a previous publication in which the reaction rates of various metabolites with 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine were described, unreacted triazine was determined colorimetrically by treating it with pyridine and alkali (1, 2). A similar procedure was found to be applicable to the analysis of FDNB which differed only in that absorption measurements were made at 548 mμ instead of 440 mμ. The red violet color which was formed faded at a rate of about 1 per cent per minute at 25° C. in contrast to rates of about 3 per cent per minute for the triazines (1).

Two methods were used for stopping the reactions in the earlier investigations. In method *A* the pyridine reagent was added directly to the reaction mixture, and the color developed in the presence of buffer and excess substrate. In method *B* the unreacted triazine was extracted from the aqueous phase with petroleum ether and the triazine determined colorimetrically after evaporation of the solvent. Method *A* was not applicable to measuring the reaction rates of the *s*-triazine with glutathione and certain other metabolites, and similar difficulties were experienced with FDNB because of interferences from some of the substrates.

In view of this, the extraction procedure was used throughout to provide for uniform treatment. However the solubility of FDNB in aqueous buffer was found to be about 400 μg. per gram compared to 10 μg. per gram for the *s*-triazine so that quantitative separation from the aqueous phase could not be obtained by a single extraction with benzene or petroleum ether. Dichloromethane was found to give satisfactory results and was used for the extraction of both toxicants. About 0.08 ml. of tetraethylene glycol dimethyl ether was added as a fixative to prevent losses of FDNB by volatilization during removal of the solvent. This change in technique resulted in the isolation of the alkylating agents from buffer, excess substrate and intermediate reaction products so that the analytical determinations were free from interferences. The velocity constant for the reaction of the *s*-triazine with glycine was previously reported to be 3.5 mole liter^{-1}sec.$^{-1}$ compared to a value of 3.0 mole liter^{-1}sec.$^{-1}$ obtained by the revised procedure.

Within a single experiment the mean velocity coefficient could be determined with an accuracy of 1 per cent. In independent experiments reproducibility was much poorer because reaction rates with most substrates changed 10-fold with each unit change in pH. The Model G Beckman pH meter used in these investigations was warranted to give results with an accuracy of 0.05 pH unit while the standard buffers had a pH of 7.00 ± 0.02 pH units. Thus fluctuations in instrument readings and buffer alone could lead to errors of 20 and 10 per cent respectively. In actual practice the coefficient of variation was found to be 5 per cent using the same equipment so it is evident that precision of measurement was considerably better than absolute accuracy. All rate measurements were made at least twice in independent experiments conducted on different days, and determinations repeated in cases where the results did not agree to within 5 per cent.

REACTION RATES

FDNB was found to alkylate the amino acid glycine only about 10 per cent as fast as the *s*-triazine so experiments with this compound were carried out at pH 8.0 instead of pH 7.0. This resulted in a reduction of the half-time for the reaction from about 690 minutes to 68 minutes so that results could be obtained in a convenient length of time without using excessive concentrations of substrate. The modified second order rate equation (2) previously used to correct the observed reaction rates of the *s*-triazine for the pH of the medium and the dissociation constants of the functional groups of the substrates[1] was equally applicable to the reactions of FDNB with various metabolites (Table I). Thus the apparent rate constants of compounds containing primary amino, secondary amino, phenolic and sulfhydryl groups increased about 10-fold when the pH was increased from

TABLE I

Reactivities of Metabolites with FDNB at Two pH Values

Compound	pK′	Apparent rate constant $\times 10^3 (k)$	
		pH 7.0	pH 8.0
Proline	10.6	3.6	43
Glycine	9.60	0.68	6.3
Glutathione	9.12*	970.	11,000
p-Aminobenzoic acid	4.92	6.7	6.7
Nicotinic acid	4.87	3.3	3.1
p-Hydroxybenzoic acid	9.40	1.3	14

* pH at which $[S^-] = [SH]$.

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[1] The absolute accuracy of the intrinsic velocity constants is limited by the fact that they were calculated using apparent dissociation constants obtained under different conditions of temperature and ionic strength than the rate measurements (2).

7.0 to 8.0 while rates of reaction with compounds having pK′ values considerably below 7.0 such as *p*-aminobenzoic and nicotinic acids remained approximately constant. Except for these latter compounds, the apparent rate constants are incorrect since erroneous values were used for the concentrations of the substrates. It is therefore necessary to define the apparent rate constant as

$$k = \frac{k'K'}{K' + a_{H^+}}$$

where K' is the apparent dissociation constant of the amino or sulfhydryl group participating in the reaction and a_{H^+} is the hydrogen ion activity of the medium. The constant k' may be a composite one since different forms such as

$$\left[\begin{matrix}NH_2\\COO^-\end{matrix}\right. \quad \text{and} \quad \left[\begin{matrix}NH_2\\COOH\end{matrix}\right.$$

may react at different rates. However, under the conditions described here it is evident that the former predominates.

Even though values of k are not true constants, they are useful for comparing the reactivities of compounds in solutions having the same pH values and approximately equivalent ionic strengths, since, in practice, degree of dissociation as well as intrinsic reactivity determines which of two materials will react at the faster rate. For example, the intrinsic reactivities of the hydroxyl groups of serine and threonine are probably very high, but they cannot combine with the *s*-triazine at appreciable rates in the physiological pH range because they do not ionize.

On comparing the apparent reactivities of FDNB and the *s*-triazine with compounds containing primary amino groups (Table II) at pH 7.0, it

TABLE II

COMPARISON OF THE SECOND ORDER VELOCITY COEFFICIENTS (MOLE LITER^{-1} SEC.$^{-1}$) FOR THE REACTIONS OF FDNB AND *s*-TRIAZINE WITH COMPOUNDS CONTAINING PRIMARY AMINO GROUPS

Compound	pK′	Rate constant		Apparent constant ×10³ at pH 7.0	
		s-Triazine	FDNB	*s*-Triazine	FDNB*
γ-Aminobutyric acid	10.43	7.0	0.43	2.6	0.16
Ethylamine	10.67	5.7	0.35	1.2	0.072
β-Alanine	10.19	4.0	0.36	2.6	0.23
Glycine	9.60	3.0	0.27	7.6	0.63
Leucine	9.60	1.8	0.19	4.5	0.47
α-Alanine	9.69	1.1	0.12	2.1	0.24
Taurine	8.74	1.1	0.23	20.	1.3

* Calculated from data at pH 8.0.

was found that the FDNB reacted much more slowly in all cases. The order of apparent reactivity with the *s*-triazine was taurine > glycine > leucine > γ-aminobutyric acid = β-alanine > α-alanine > ethylamine, while for FDNB it was taurine > glycine > leucine > α-alanine = β-alanine > γ-aminobutyric acid > ethylamine. Some minor inversions in order occurred but in general reactivity with FDNB responded in the same way with respect to structural changes as the *s*-triazine. The correlation coefficient obtained on comparing reactivities with the two toxicants was 0.834 which is significant at the 1 per cent level. When velocity coefficients were corrected for the dissociation constants of the functional groups the order of reactivity with the *s*-triazine was found to be γ-aminobutyric acid > ethylamine > β-alanine > glycine > leucine > α-alanine = taurine. Thus some of the compounds which possessed the highest apparent reactivities had the lowest intrinsic reactivities and vice versa. The intrinsic reactivity of the primary amino group decreased with proximity to a carboxyl group while its ionization increased. Compounds with terminal amino groups which did not contain a carboxyl group or were separated from it by one or more methylene groups were more reactive intrinsically than glycine. This was probably an inductive effect.

The order of intrinsic reactivity was about the same for FDNB as for the *s*-triazine although again the absolute values were much lower. The only inversion of any consequence occurred in the case of taurine which had intermediate reactivity with FDNB and low reactivity with the triazine. These comparisons showed that the correlation observed between the reactivities of the two compounds was not caused entirely by differences in the dissociation constants of the substrates, since they responded in the same general way to steric effects and changes in nucleophilicities of the functional groups.

Much larger differences were found when reactivities with compounds containing different functional groups were compared. Of greatest interest was the fact that the two toxicants reacted at about the same rate with glutathione while FDNB combined only about 10 per cent as fast as the *s*-triazine with compounds containing primary amino groups (Table III). The reactivity of FDNB with proline and *p*-hydroxybenzoic acid was also very low in comparison to glutathione. However the largest reduction in relative reactivity occurred with *p*-aminobenzoic and nicotinic acids. The former compound combined with the *s*-triazine about one-third as fast as glutathione while in the case of FDNB it was only about 1 per cent as reactive. Thus the high susceptibility of these compounds to attack by the *s*-triazines which was caused by their low pK′ values was not found in the case of FDNB because of very low intrinsic reactivities.

When velocity coefficients were corrected for the pK' values of the functional groups intrinsic reactivities decreased in the same general order

TABLE III

COMPARISON OF THE APPARENT AND CORRECTED RATE CONSTANTS FOR THE REACTION OF *s*-TRIAZINE AND FDNB WITH COMPOUNDS CONTAINING VARIOUS FUNCTIONAL GROUPS

Compound	pK′	Rate constant (mole liter^{-1} sec.$^{-1}$)		Apparent rate × 10^3 at pH 7.0 (mole liter^{-1} sec.$^{-1}$)	
		s-Triazine	FDNB	*s*-Triazine	FDNB
Glutathione	9.12	140	130	1,100	970
Proline	10.6	350	16	87	3.6
p-Hydroxybenzoic acid	9.40	7.0	0.31	28	1.3
$R\text{-}NH_2$ (average)	—	2.8	0.25	6.3	0.6*
p-Aminobenzoic acid	4.92	0.32	0.0067	320	6.7
Nicotinic acid	4.87	0.099	0.0033	99	3.3

* Calculated from data at pH 8.0.

for the two compounds from glutathione to nicotinic acid, except that the dissociated secondary amino group of proline was more susceptible to attack by the *s*-triazine than the $R\text{-}S^-$ group of glutathione while the reverse was true for FDNB. Furthermore some of the quantitative differences were very large, so even though the two compounds paralleled one another in reactivity in some respects, they differed considerably in their specificities for various functional groups.

DISCUSSION

These measurements were made at one temperature only, so it is not known whether the observed differences in reactivities were caused by changes in the frequency factor or by changes in the free energies and entropies of activation. Chapman *et al.* (4, 5) studied the kinetics of the reaction of FDNB with primary aromatic amines in alcohol solutions and found that the energy of activation was about 5,000 calories less than for the chlorine analogue. He also found that only one mole of amine was consumed per mole of FDNB which indicated that $R\text{-}NH_3F$ was reactive. On this basis he proposed that the transition state was more highly solvated when the halogen was fluorine rather than chlorine because of the small size of the fluoride ion and its comparatively large solvation energy. However in these present investigations the rates were measured in aqueous media and were found to be pH dependent for substrates having high *pK′* values. This shows that FDNB is incapable of reacting with $R\text{-}NH_3^+$ even though it may be able to combine with $R\text{-}NH_3F$ in ethanol.

From the standpoint of toxicology, the most interesting observation made during the course of this work is the finding that FDNB is a more specific reagent for sulfhydryl groups than the *s*-triazine. Thus, if the apparent rate constants (Table III) for the six functional groups that were investigated are given equal weight, it is found that sulfhydryl reactivity accounts for 98 per cent of their sum for FDNB and only 67 per cent for the *s*-triazine. Furthermore, the *s*-triazine is highly reactive with *p*-aminobenzoic and nicotinic acids because of their low dissociation constants while much of this effect is lost with FDNB. These differences in group specificity may explain in part why FDNB is about 50 times as effective as the *s*-triazine on a weight basis for preventing the damping-off of pea seedlings by soil borne fungi[2], for in the soil it is unlikely that large concentrations of sulfhydryl compounds will accumulate in view of the ease with which they oxidize. However, organic amines and phenolic compounds may occur in comparatively large amounts through the decomposition of organic matter. In the soil environment FDNB would be expected to be considerably more stable than the *s*-triazine since it reacts with these compounds at much slower rates, but once it were accumulated in fungus mycelium it would be able to react with essential sulfhydryl groups at about the same rate as the *s*-triazine and with a much higher degree of specificity. Thus stability in the soil environment might give FDNB an advantage in this application.

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INTRACELLULAR DISTRIBUTION OF METAL IONS AND ORGANIC FUNGICIDES IN FUNGUS SPORES[1]

Robert G. Owens and Lawrence P. Miller

SUMMARY

Conidia of *Neurospora sitophila* and *Aspergillus niger* were disintegrated by sonic treatment after exposure to sublethal amounts of dichlone-C^{14}, glyodin-C^{14} base, Ag^{110}, Ce^{144}, Zn^{65}, Cd^{115}, or Hg^{203}. The homogenate after sonic treatment was partitioned by differential centrifugation into cell walls, two particulate fractions and a water-soluble fraction. The distribution of organic fungicides and metal ions among the different fractions was then determined from radioactivity values. All toxicants except dichlone were found to accumulate to higher levels in at least one of the particulate fractions than in the spore as a whole on the basis of equivalent cell solids. Little, if any, of the toxicants accumulated in the cell walls. More than 75 per cent of the glyodin base in conidia of *N. sitophila* was found to be associated with small cellular particles which sedimented after one hour at 80,000×g. About 75 per cent of the dichlone was found in the supernatant solution. Fifteen per cent of the dichlone in the supernatant was associated with soluble proteins and 60 per cent was associated with acetone and water-soluble components or occurred as unaltered dichlone. Some differences in distribution patterns for *A. niger* and *N. sitophila* spores were found. Cerium accumulated to higher levels in the larger particles of *A. niger* spores than in either particulate fraction of *N. sitophila* spores. More silver occurred in the supernatant solution from spores of *A. niger* than from those of *N. sitophila*. About 40 per cent of the dichlone and silver in particles from *N. sitophila* spores could be removed either by sonic treatment or by extraction with acetone and 5 per cent HCl. The remainder was apparently firmly bound or reacted with structural components of the particles.

The data are compatible with previous convictions that fungicides are absorbed by fungus spores and that sufficient toxicant accumulates in cytoplasmic particles to inactivate many kinds of enzymes associated with the particles.

INTRODUCTION

A considerable amount of work has been done recently relating the toxic action of organic fungicides and heavy metal ions to inhibition of enzymes. The assumption is made that most of the intermediary metabolism of spores occurs within the cytoplasm and that fungicides are taken into the cytoplasm. Occasionally, however, the question is raised whether fungicides penetrate into the cytoplasm or whether they exert a toxic effect through some kind of action at the cytoplasmic surface or on the cytoplasmic membrane itself. Some evidence exists which shows that several organic fungicides and metal ions do affect the cytoplasmic membrane, causing leaching of cell contents into the ambient solution (1, 3). In many

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[1] This work was supported in part by a grant from the Rockefeller Foundation.

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cases, however, some leaching may occur at sublethal concentrations of toxicants.

There is also substantial direct evidence that toxicants enter the cytoplasm concomitant with any effects they may have on the cytoplasmic membrane. Mudd and Anderson (5), for example, have shown that silver becomes located in the cytoplasm of bacteria and is not retained at the cell surface. Tröger (9) was able to detect copper ions within the cytoplasm of conidia of *Fusarium decemcellulare* after suspending the spores in a solution of copper sulfate, and McCallan and Miller (2) cite other examples of earlier demonstrations of the presence of copper and mercury within the cytoplasm of fungi and bacteria.

Although the work referred to provides ample proof that toxicants can enter the cytoplasm, the question of whether or not they become located in vital areas in the quantities necessary to account for inhibition of enzymes has not been resolved. Since present investigations on fungicidal mechanisms deal largely with inhibition of intracellular enzymes, some more direct quantitative evidence of absorption and intracellular accumulation of fungicides was deemed necessary. This paper reports results from a study made to determine the general areas and amounts of accumulation of dichlone, glyodin base and several metal ions in fungus spores. A preliminary account of these results has appeared elsewhere in abstract form (7).

MATERIALS AND METHODS

Conidia of *Neurospora sitophila* (Mont.) Shear & Dodge and *Aspergillus niger* van Tiegh were used in these experiments. *N. sitophila* was cultured as described previously (6). *A. niger* was cultured on boiled wheat grains in 250-ml. Erlenmeyer flasks. The conidia were filtered through a double layer of cheesecloth to remove mycelium and washed by repeated suspension and sedimentation in 15-ml. centrifuge tubes in a clinical centrifuge. Washing was continued, with changes of water after each centrifugation, until the supernatant solution remained clear. The washed spores were brought to a known volume with distilled water and suspended homogeneously with a mechanical stirrer. Two or more 2-ml. samples were transferred to preweighed 5-ml. crucibles and dried for six hours in an oven at 100° C. and reweighed. The dry weight of the spores thus obtained is used as the basis for expressing all results. Dry weights of each spore fraction were obtained by drying and weighing suitable samples in weighing bottles.

Between 150 and 250 mg. of spores in 15-ml. centrifuge tubes were exposed to sublethal amounts of C^{14}-labeled dichlone (2,3-dichloro-1,4-naphthoquinone) and glyodin base (2-heptadecyl-2-imidazoline), Ag^{110}-labeled $AgNO_3$, Ce^{144}-labeled $CeSO_4$, and Zn^{65}-, Cd^{115}- and Hg^{203}-labeled acetates in 10 ml. of water. Exposure periods varied up to one hour. No attempt was made to keep exposure time constant because the data are based on radioactivity retained by the spores. After exposure the spores were washed by centrifuging in several changes of distilled water until radioactivity of the wash water became negligible. They were then transferred quantitatively in 30 ml. of distilled water to the treatment cup of a Raytheon 250 W., 10 Kc. Magnetostrictive Sonic Oscillator, Model DF-101.[2] This apparatus is equipped with a water jacket around the sample cup and cold water was circulated through the jacket to prevent heating which normally accompanies sonic treatment of solutions at high frequencies. The spores were treated for three hours or more, depending upon the progress of disintegration. After disintegration, spore walls and any remaining intact spores were separated from cytoplasmic components by centrifuging the homogenate for five minutes at 500×g. Spore walls, designated fraction *W*, were washed by recentrifuging several times in fresh water to remove adhering cytoplasmic material. Washings were combined with the spore- and wall-free homogenate and then the whole homogenate was partitioned in a Spinco ultracentrifuge into three fractions: (P_1) particles sedimenting in 30 minutes at 10,000×g., (P_2) particles sedimenting in one hour at 80,000×g. after removal of fraction P_1, and (S) the supernatant solution after removal of fraction P_2. The spore walls and particulate fractions P_1 and P_2 were taken up separately in a known volume of distilled water. Aliquots containing less than one mg. of solid from each suspension were transferred to nickel-plated planchets one inch in diameter and dried under an infrared lamp. The radioactivity was then determined with the use of a thin end-window Geiger tube, conventional scaling equipment, and an automatic sample changer. The equipment and procedure for counting have been described previously (4). The weight of toxicant in each fraction was calculated from the radioactivity with reference to appropriate standards.

In determining the release of toxicants from cytoplasmic particles caused by sonic treatment, conidia exposed to dichlone-C^{14} or Ag^{110} were disintegrated as described above. The cell walls and intact spores were removed and the homogenate was centrifuged for 30 minutes at 10,000×g. The supernatant from this centrifugation was discarded. The particulate pellet containing radioactive toxicant was saved. An equal quantity of nonexposed conidia was disintegrated and cell walls were removed as before. The homogenate was centrifuged for 30 minutes at 12,000×g. The supernatant solution from this centrifugation was saved and the particulate pellet was discarded. The supernatant was mixed with the particles from conidia exposed to the toxicant and the particles were subjected to sonic treatment for 30 minutes in the oscillator cup. The homogenate was then centrifuged for 30 minutes at 12,000×g. to sediment the particles which originally contained all of the radioactivity. The supernatant solution was decanted and centrifuged for one hour at 80,000×g. to sediment the lighter particles. This gave three fractions: (a) the heavier particles which originally contained all of the radioactivity, (b) lighter particles which originally contained no radioactivity, and (c) a supernatant solution which originally contained no radioactivity. Aliquots of each fraction were transferred to planchets, dried, and the amount of toxicant in each fraction determined as described above.

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[2] Raytheon Manufacturing Company, Waltham 54, Massachusetts.

RESULTS

DISINTEGRATION OF SPORES

Initially several methods were tried for disintegrating fungus spores, including use of various types of hand and mechanical homogenizers and grinding with abrasives in a mortar. None gave satisfactory disintegration, and grinding with abrasives had the added disadvantage of contamination with abrasive particles that could not be separated quantitatively from the spore contents. Subsequent trials with sonic waves were more encouraging. The Raytheon 250 W., 10 Kc. Sonic Oscillator was found to give excellent disintegration of conidia of *N. sitophila*. Preliminary tests showed that more than 90 per cent of the conidia in a 10 per cent aqueous suspension (v/v) were disintegrated in two hours. Breakage of the conidia, determined by periodic counts of remaining intact conidia, proceeded logarithmically and was dependent upon the number of intact spores remaining. This relationship can be expressed in terms of the law of mass action where only one component is decreasing, i.e.,

$$kt = 2.303 \log \frac{C_o}{C_t}$$

where k is a constant of proportionality, t is time, C_o is the number of spores present at zero time, and C_t is the number of spores present after time t. According to this equation $\log C_o/C_t$ should be proportional to time and give a straight line passing through the origin when plotted as a function of time. That this relationship holds is shown by the fit of points in Figure 1. However, the line does not pass through the origin probably indicating that there is a short lag period before disintegration begins.

DISTRIBUTION OF TOXICANTS IN VARIOUS FRACTIONS OF SPORES

The average percentage weight distribution of each fraction of spores of *N. sitophila* is given along with other data in Table I. Spore walls, W, and the supernatant fraction, S, contained a larger amount of solids than did either particulate fraction alone. However, the weight of the two particulate fractions combined was about equal to that of the cell walls or supernatant solids.

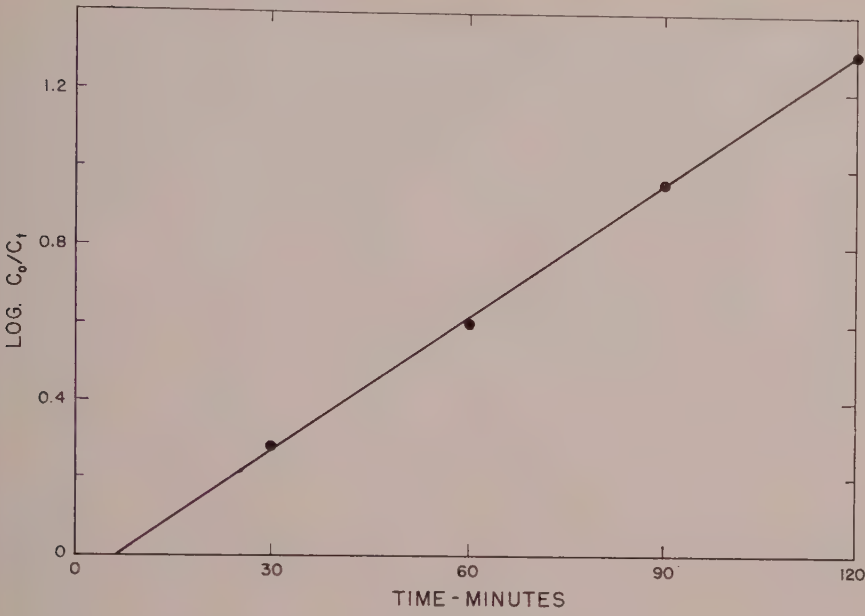

FIGURE 1. Semilogarithmic plot of disintegration of conidia of *Neurospora sitophila* by sonic treatment in relation to time. C_o is the initial concentration of spores and C_t is the concentration after time *t*.

Despite the fact that cell walls made up 35 per cent of the total spore weight, only 7 per cent or less of any toxicant was retained by the walls. Apparently even this small percentage was due primarily to residual intact spores that were sedimented along with the spore walls. All of the toxicants were absorbed to some extent by particulate fractions P_1 and P_2 and, with the exception of dichlone, reached higher levels in at least one of these fractions than in the whole spores when calculated on the basis of equivalent amounts of cell solids, as is shown in part 3 of Table I.

Cerium, cadmium and zinc were almost equally distributed among fractions P_1, P_2 and S on the basis of equivalent cell solids, as is shown by the ratio of toxicant per gram of solid in each of these fractions to toxicant per gram of spores, part 3, Table I. Silver and mercury accumulated principally in fractions P_1 and P_2, although a considerable amount of silver was present also in fraction S. The bulk of glyodin base was found in fraction P_2, with small amounts present also in fractions P_1 and S. More than 75 per cent of the total glyodin base in fraction P_2 was associated with lipids and could be extracted with acetone. The remaining portion, however, appeared to be strongly bound to nonlipid components.

Dichlone was found principally in the supernatant solution. Less than 25 per cent of the total was in the particles. Of that amount in the particles, 58 per cent was strongly bound to acetone- and acid-insoluble components.

TABLE I

DISTRIBUTION OF METAL IONS AND ORGANIC FUNGICIDES IN CONIDIA OF NEUROSPORA SITOPHILA

Toxicant	Toxicant, µg./g. spores in				
	Whole spores	Spore walls (W)	Particulate fraction sedimented at		Supernatant (S)
			10,000×g. (P_1)	80,000×g. (P_2)	
Cerium	710	25	75	320	290
Silver	185	5	45	85	50
Mercury	1285	0	460	820	5
Cadmium	45	0	5	15	25
Zinc	205	15	20	70	100
Glyodin base	765	25	40	585	115
Dichlone	530	5	25	100	400
Distribution as per cent of total toxicant					
Cerium		4	10	45	41
Silver		4	25	45	26
Mercury		0	36	63	1
Cadmium		0	14	35	51
Zinc		7	10	34	49
Glyodin base		3	5	77	15
Dichlone		2	4	19	75
Ratio of toxicant per g. fraction/toxicant per g. spores					
Cerium		0.1	1.2	1.6	1.3
Silver		0.1	5.0	2.2	0.8
Mercury		0	2.4	2.6	0.1
Cadmium		0	1.6	1.5	1.5
Zinc		0.2	2.0	1.5	1.4
Glyodin base		0.1	0.6	3.2	0.5
Dichlone		0.1	0.5	0.8	2.3
Average weight distribution—%		35	9	24	32

Extraction of the particles with acetone removed 22 per cent of the dichlone and subsequent extraction with 5 per cent HCl yielded an additional 20 per cent.

Since such a large part of the total dichlone appeared in the supernatant solution, it was of interest to determine whether or not it was associated with soluble proteins. The proteins were precipitated by addition of nine volumes of acetone. Aliquots of the solution suitable for radioactivity determinations were taken before and after precipitation. It was found that removal of the proteins resulted in a decrease of about 15 per cent in radioactivity of the solution. Thus about 85 per cent of the dichlone was associated with acetone and water-soluble components or occurred as unaltered dichlone.

Conidia of *A. niger* were not disintegrated as nearly quantitatively as spores of *N. sitophila* even though they were treated sonically two to three hours longer. Disintegration varied from about 90 per cent down to little more than 50 per cent in some cases. Since the spore walls and residual intact spores could not be separated the amount of retention of toxicant, if any, by the walls could not be determined. Therefore uptake values obtained on fraction *W* of *A. niger* spores had little meaning with regard to uptake by the walls and are omitted. Moreover, the values obtained for fractions P_1, P_2 and S obviously are not quantitative because they represent only a portion of the total amount of solids in the original spores. However, the values for each of these three fractions are relative and do provide some insight into the distribution patterns of toxicants in *A. niger* spores (Table II).

TABLE II

DISTRIBUTION OF METAL IONS AND ORGANIC FUNGICIDES IN CONIDIA OF ASPERGILLUS NIGER

Toxicant	Toxicant, µg./g. spores in			
	Whole spores	Particulate fraction sedimented at		Supernatant (S)
		10,000×g. (P_1)	80,000×g. (P_2)	
Cerium	5090	2440	335	170
Silver	505	145	75	195
Mercury	6420	3100	525	735
Cadmium	5195	1820	280	565
Glyodin base	770	445	240	50
Dichlone	1240	170	13	960
Distribution as per cent of total toxicant				
Cerium		42	7	3
Silver		29	14	39
Mercury		48	8	12
Cadmium		35	6	11
Glyodin base		58	16	6
Dichlone		14	2	78
Ratio of toxicant per g. fraction/toxicant per g. spores				
Cerium		1.9	0.8	0.1
Silver		1.2	0.1	1.5
Mercury		1.0	0.7	0.2
Cadmium		1.1	0.5	0.3
Glyodin base		2.3	1.8	0.2
Dichlone		0.5	0.2	2.6
Average weight distribution—%		26	9	30

The average weight distribution of solids was somewhat different from that for *N. sitophila* spores. The larger portion of cytoplasmic particles in

A. niger sedimented at 10,000×g. and a smaller portion at 80,000×g. The reverse was true for *N. sitophila* spores (compare Tables I and II). Solids appearing in the supernatant solution made up about the same proportion of total solids in spores of both fungi.

In *A. niger* spores the metal ions, with the exception of silver ions, tended to accumulate more in fraction P_1 than in P_2 (part 3, Table II). A large portion of silver was found in S, whereas it accumulated to a very high concentration in fraction P_1 in *N. sitophila* conidia. The distribution of cerium also was notably different in conidia of the two fungi. In *A. niger* all but 10 per cent of the amount recovered was present in fraction P_1. In *N. sitophila* it was distributed equally among P_1, P_2 and S on the basis of equivalent weights of solids.

Glyodin base accumulated to about the same extent in both particulate fractions in *A. niger* conidia, whereas in *N. sitophila* conidia it occurred predominantly in fraction P_2. Dichlone was found in the supernatant solution in essentially the same proportion in spores of both fungi.

RELEASE AND REABSORPTION OF DICHLONE AND SILVER BY CYTOPLASMIC PARTICLES SUBJECTED TO SONIC VIBRATIONS

In pretreating spores with toxicants and subsequently disintegrating them by sonic treatment, there was a question whether the toxicant remained with the same cell component with which it was originally associated in the intact spores. Therefore a test was devised to determine to what extent dichlone and silver are released from cytoplasmic particles by sonic treatment. The results are given in Table III. Both dichlone and silver which had been contained only in particulate fraction P_1 were found in fractions P_2 and S. However, more than 50 per cent of both toxicants was re-

TABLE III

RELEASE AND REABSORPTION OF DICHLONE-C^{14} AND Ag^{110} FROM CYTOPLASMIC PARTICLES FROM CONIDIA OF NEUROSPORA SITOPHILA BY SONIC TREATMENT

Fractions	Percentage of total	
	Dichlone	Silver
A. Particles sedimented at 10,000×g. from spores exposed to toxicant	100	100
B. Supernatant from untreated spores after removal of particles sedimenting at 12,000×g.	0	0
C. Mixture of A+B	100	100
D. Particles from C mixture resedimented at 12,000 ×g. after sonic treatment (P_1)	56	61
E. Particles from C mixture sedimented at 80,000×g. after sonic treatment (P_2)	25	31
F. Supernatant from C mixture after centrifuging at 80,000×g. after sonic treatment (S)	19	8

tained by the original particles even when the particles were mixed with more than five times their weight of cell solids from untreated spores. Thus while a portion of the toxicant in the particles was bound loosely and could be released and reabsorbed by fresh particles, a large portion of it was firmly bound. This is in accord with the results obtained by extracting the particles with acetone and 5 per cent HCl. Use of either sonic waves or solvents to remove the dichlone resulted in removal of less than 50 per cent of the total amount present. The remainder was bound in such a way that it could not be removed without complete degradation of the particles. The data indicate that while the values obtained for toxicants in each fraction in the present tests do not precisely represent the situation in intact spores, it is reasonable to expect that there was more rather than less toxicant in particulate fractions within intact spores, since sonic treatment tended to cause release of toxicants into the water.

DISCUSSION

Partition of spore components into fractions sedimenting at 10,000×g. and at 80,000×g. was based on procedures used for separation of mitochondria and microsomes of other organisms. It has not been shown, however, that the two fractions from spores can properly be designated as mitochondria and microsomes. On the other hand, it is apparent from the large differences in the amounts of glyodin base in fraction S, P_1 and P_2 from spores of *N. sitophila* that some fundamental difference in the composition of the two fractions exists, at least in the case of *N. sitophila* spores. A similar difference was not found for *A. niger* spores, which would rule out the possibility that the difference might be due to greater specific surface of the smaller particles.

The data from experiments with spores of *N. sitophila*, which were almost quantitative, show clearly that cytoplasmic contents of fungus spores have a great affinity for metal ions, dichlone and glyodin base and that spore walls have little or no affinity for these materials. Except for dichlone, all of the toxicants accumulated to a greater extent in cytoplasmic particles than in the spore as a whole on the basis of equivalent cell solids. For cerium, zinc and cadmium, this was due largely to the fact that the walls, which made up 35 per cent of the spore weight, retained little of the ions. For mercury, silver and glyodin base, the accumulation was partly at the expense of the supernatant fraction also.

The distribution of dichlone was notably different from that of other materials. It reacted to some extent with components in the cytoplasmic particles and with proteins in the supernatant solution but about 60 per cent of it was associated with acetone and water-soluble constituents in the supernatant solution. These results are in accord with unpublished findings in current studies on the mechanism of action of dichlone. It has been found that a large portion of dichlone incubated aerobically with conidia of *N. sitophila* reacts with substances from the spores to form brown and yellow water-soluble products. At least five such products have been separated but not yet identified. These products are formed rapidly and at concentrations of dichlone well below those required to inhibit germination of the conidia.

In other organisms particulate components of cells, such as mitochondria and microsomes, have been shown to possess various kinds of enzymatic activities (8). The present data show that toxicants accumulate in these areas well within the range of quantities necessary to inhibit many enzymes. For example, it is not uncommon for solutions containing as little as 1 to 10 micrograms of toxicant per ml. (or gram) of water to inhibit unprotected enzymes *in vitro*. All of the toxicants tested accumulated to the extent of hundreds or thousands of micrograms per gram of cell solids. The fact that such high concentrations did not prevent germination of the spores in the present tests argues strongly that the enzymes were protected through indiscriminate interaction of the toxicants with substances other than enzymes. These nonspecific interactions very likely account for the large amounts of fungicides shown to be required for inhibition of germination of fungus spores (2).

While the present data are in general accord with previous work showing that certain toxicants are absorbed by spores (2, 5, 9), the data on redistribution from particles containing dichlone or silver to fresh particles are difficult to interpret, since the effects of sonic waves on the particles are unknown. It is possible that vibration at high frequency tends to fragment some of the larger particles in which case the fragments might appear in the fraction with the smaller particles. It is possible also that there is a gradual erosion of molecules from the particles so that part of the material in the particles appears in the supernatant solution after sonic treatment. If, however, the data represent true re-distribution, they indicate that an important part of the toxicants are susceptible to exchange. Thus it might be argued that the toxicant was located at the cytoplasmic surface until the spores were disintegrated and that the distribution patterns obtained reflect only the relative affinity of the toxicants for components of the various fractions when no plasma membrane is present. While this argument is not without merit it would be difficult to understand the nature of the affinity which resulted in such large accumulation of toxicants, excluding them from contact with internal components in the intact spore but yielding them readily after disintegration. This argument assumes also that the surface imparts all of the affinity exhibited by spores for the toxicants. However data by Miller *et al.* (4) on uptake of silver, cerium and glyodin base by spores of *N. sitophila* show that the relative order of affinity is glyodin base > silver > cerium, which is the same order of affinity shown by particles in part 2 of Table I. Both particulate fractions combined contained 82, 70 and 55 per cent of the glyodin base, silver and cerium, respectively. The affinity of *A. niger* spores for silver and cerium is reversed from that of *N. sitophila* spores (4). The order is also reversed in case of the particles (Table II). This suggests that the particles contribute to the affinity exhibited by the whole spores for various toxicants.

Perhaps the strongest evidence against the exclusion of toxicants by the plasma membrane consists in the fact that enzyme studies as yet unpublished show that enzymes commonly associated with cellular particles are inactivated in intact spores by fungicides and heavy metal ions. The magnitude of accumulation of toxicants in cytoplasmic particles in the present study is compatible with the studies on enzymes associated with the particles.

It is concluded that the toxicants with which this paper is concerned are absorbed by spores and not retained at the spore surface. Apparently a considerable portion of these toxicants are bound by cytoplasmic components which are not directly involved in metabolism. This results in removal of large quantities of toxicant from solution before viability of the spores is greatly reduced and also accounts for the fact that spores will continue to absorb the toxicants even after lethal amounts have been taken up. It is recognized also that some chemicals reduce viability of spores without being bound in large quantities and in some cases, such as with copper (2), transfer of the spores to a toxicant-free medium results in leaching of the toxicants in sufficient quantities to allow germination to proceed. For such chemicals it seems likely that the interaction between the toxicant and cell components, including essential enzymes, is concentration dependent and readily reversible when the concentration is diminished beyond a critical level.

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THE SULFUR METABOLISM OF INSECTS. II. THE METABOLISM OF CYSTINE-S^{35} BY THE HOUSE FLY, MUSCA DOMESTICA L.[1]

John D. Hilchey,[2] Val F. Cotty,[3] and S. Mark Henry

SUMMARY

Cystine-S^{35} fed to adult male or female house flies, *Musca domestica* L., was converted into taurine and sulfate. Taurine was partly retained in the body and partly excreted while virtually all of the sulfate was excreted within 24 to 48 hours after it was formed. Cystine was not transformed into methionine.

INTRODUCTION

Sulfur compounds play important roles in many phases of metabolism in vertebrates. Very little, however, is known of the metabolic pathways in insects.

Nutritional studies on several species of insects have provided clues to the metabolism of sulfur containing amino acids. *Attagenus* sp. (15), *Tribolium confusum* Duv. (4, 13), *Calliphora vicina* Robineau-Desvoidy (17), *Pseudosarcophaga affinis* (Fall.) (10), *Apis mellifera* L. (3), *Chilo simplex* (Butl.) (11) and probably *Drosophila melanogaster* Meigen (8, 12) require methionine but not cystine for growth and development and are probably capable of performing transulfuration or a similar process. *Aedes aegypti* (L.) (6) *Phoenica sericata* (Meigen.) (14) and *Oryzaephilus surinamensis* (L.) (2) require methionine for growth and cystine for growth or development. *Dermestes vulpinus* F. (5) requires cystine for development. *Blattella germanica* (L.) (7, 9) or its symbiotic flora can synthesize both methionine and cystine from Na_2SO_4.

It is the purpose of this paper to present some information on the metabolism of cystine in the house fly, *Musca domestica* L.

METHODS AND MATERIALS

Day-old house flies, reared as directed for the Peet-Grady Method (16), were confined in large beakers and fed L-cystine-S^{35} in one-fourth strength skim milk (reconstituted from skim milk powder) plus 5 per cent dextrose or a solution of amino acids similar in composition to casein plus 5 per cent

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[2] Present address: Chemicals and Plastics Division, U. S. Army Quartermaster Research and Engineering Center, Natick, Massachusetts.

[3] Present address: Bristol-Myers Products Division, Research Laboratories, Hillside, New Jersey.

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dextrose. The cystine was fed at a rate of 20 μc./ml. except in a methionine-free diet wherein it was provided at 8 μc./ml. After 16 to 20 hours, the insects were fed water or 5 per cent dextrose solution for 16 hours. This procedure permitted the digestive tract to void itself of major nitrogenous digestive residues.

The flies were anesthetized with CO_2, separated according to sex and weighed. Whole fly hydrolyzates were prepared from flies which were killed with heat (100° C. for five minutes), dried, defatted with ether and pulverized. Known weights of dried fly material were sealed in ampules with 6*N* HCl (15 ml. per 100 mg.) and autoclaved for 16 hours at 15 lb. pressure. The resulting hydrolyzate was filtered, dried on a steam bath and the residue dissolved in 1 ml. of 10 per cent 2-propanol.

Ethanolic extracts were prepared by homogenizing anesthetized flies in 80 per cent ethanol containing 6 mg. of *N*-ethylmaleimide per 25 flies (*N*-ethylmaleimide reacts with sulfhydryl groups forming adducts which are not subject to the rapid oxidation characteristic of the free sulfhydryl group). The homogenates were extracted five times with 10 ml. of 80 per cent ethanol, the insoluble substances being sedimented each time by centrifugation. The five alcohol extracts were combined, evaporated to dryness on a steam bath and the solid material remaining was dissolved in 1 ml. of 10 per cent 2-propanol. The residues left after extraction were hydrolyzed in the same manner as the unextracted flies.

Feces plus regurgitum were washed from the container, evaporated and dissolved in 2 ml. of 10 per cent 2-propanol. These preparations were then chromatographed on Whatman Nos. 1 or 3 filter paper using the solvent: *tert.*-butyl alcohol, 90 per cent formic acid, and water in the proportions 14:3:3. Both ascending and descending chromatograms were prepared. Color was developed with 0.1 per cent ninhydrin in 90 per cent acetone. When larger amounts of material were chromatographed they were applied as a series of spots or as a streak along the origin of the paper. Compounds were identified by comparing their chromatographic positions with those of known compounds. Additional evidence for the identity of a compound was obtained by an isotopic dilution technique. The radioactive intermediate was eluted from chromatograms (7) and a small amount (25 to 100 mg.) of the appropriate nonlabeled compound was added. The mixture of nonradioactive carrier plus radioactive intermediate was recrystallized several times and aliquots were removed each time and their radioactivities measured. If the specific activity remained constant it was considered confirmative evidence that the nonlabeled compound was identical to the intermediate. Cystine was reprecipitated by adjusting a solution in 2*N* HCl to pH 5 with NH_4OH solution; taurine and methionine were reprecipitated by dissolving in hot water and adding ethanol to a final concentration of 90 per cent; sulfate was precipitated as barium sulfate. This barium sulfate was purified by the ethylenediaminetetraacetic acid-nickel chloride method described elsewhere (1).

Aliquots were prepared in triplicate, no more than 1 mg. being applied to a 1¼-inch planchet. The radioactivity was measured by determining the time required for 960 counts using a thin window Geiger-Müller tube. Self absorption was avoided by reducing the total solids to 0.13 mg. or less per square centimeter.

The one-dimensional chromatograms described above were sectioned into small pieces to permit the measuring of the distribution of S^{35} along the chromatogram. Each piece was placed on a planchet and counted until 960 counts were registered. These results, corrected to a common, dry-weight basis, were used to calculate the counts per minute for each section of the chromatogram. This information was used to plot histograms.

RESULTS

The distribution of S^{35} in hydrolyzates of male and female house flies fed cystine-S^{35} in skim milk is shown in Figure 1. The two radioactive peaks correspond to cystine and taurine. No radioactive methionine was found. When the cystine-S^{35} was fed in an amino acid mixture or when methionine was omitted from a diet containing cystine-S^{35}, similar results were obtained (Figs. 2 and 3).

When the flies were extracted with 80 per cent ethanol alcohol, the residues hydrolyzed, and these preparations chromatographed it was found that most of the S^{35} was removed by extraction (Fig. 4), whereas very little remained in the residues (Fig. 5).

Chromatograms of the feces from flies fed cystine-S^{35} showed the presence of cystine, taurine and sulfate as labeled end products (Fig. 6).

The chromatographic results were confirmed by isotopic dilution and recrystallization studies as shown in Tables I, II and III.

DISCUSSION

The sugar-skim milk mixture was used as a basic diet to provide information about the performance of house flies fed on the diet employed in mass rearing techniques. The results obtained with a diet made with crystalline amino acids show that synthetic amino acid mixtures can be substituted for complete proteins for studies in which it is necessary to make systematic alterations in the quantitative relationships of the amino acids in order to determine their effect on cystine utilization by house flies.

Using these diets it was found that when cystine was fed to house flies, it appeared in the body after absorption from the digestive tract. Moreover, in all cases where cystine was fed, taurine was produced in large quantities. The cystine and taurine found in the flies, within 24 to 48 hours after feeding time, occurred almost entirely in the free form. The results with cystine

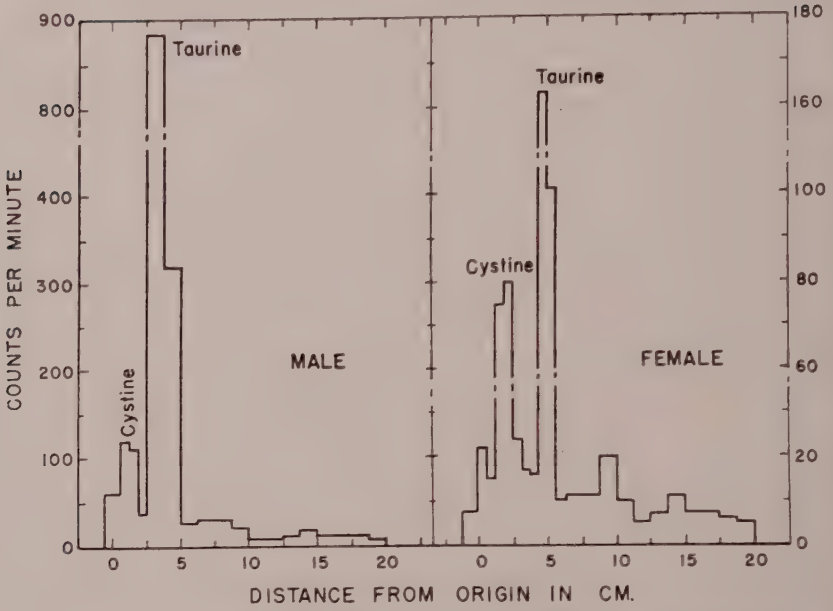

FIGURE 1. Location of radioactive substances on chromatograms of hydrolyzates of house flies fed skim milk solution containing cystine-S^{35}.

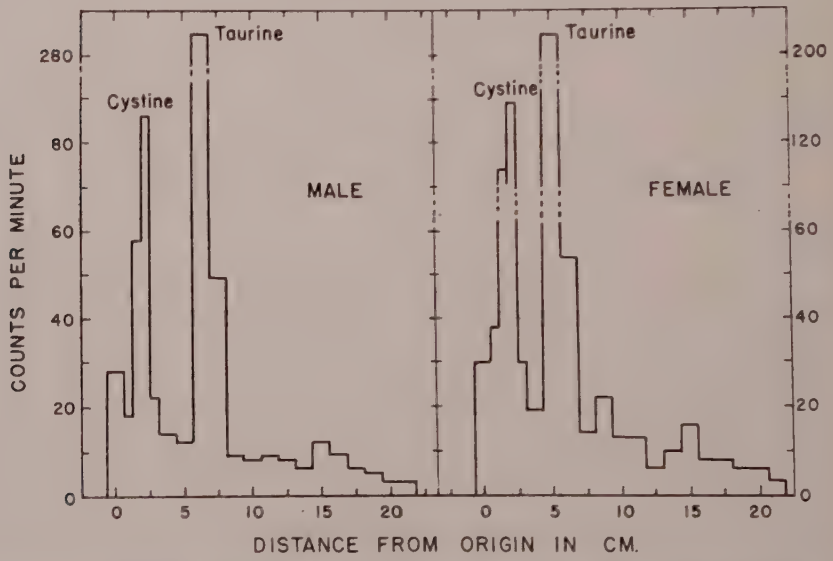

FIGURE 2. Location of radioactive substances on chromatograms of hydrolyzates of house flies fed an amino acid diet containing cystine-S^{35} and methionine.

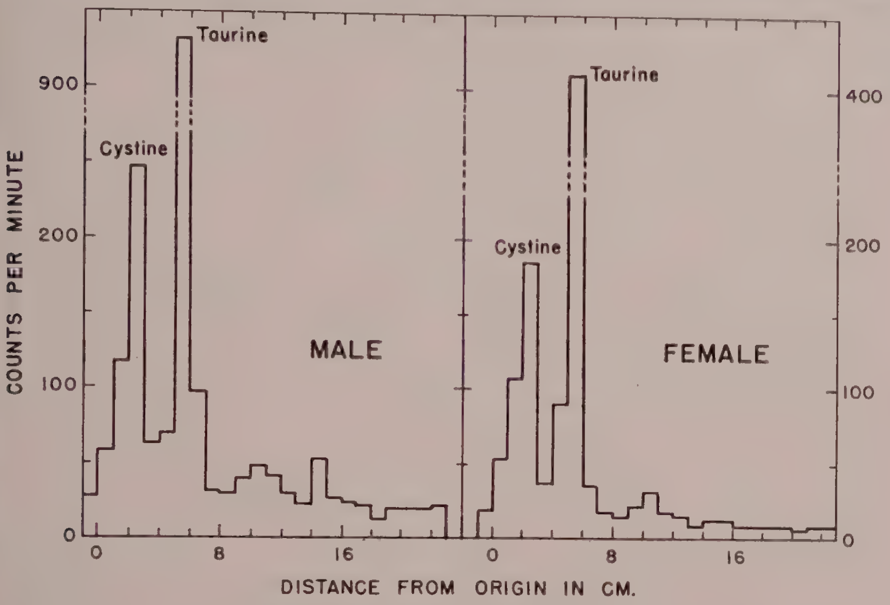

FIGURE 3. Location of radioactive substances on chromatograms of hydrolyzates of house flies fed an amino acid diet containing cystine-S^{35} but lacking methionine.

were somewhat surprising when one considers the well-known needs of other animals for the incorporation of cystine into structural and enzymatic proteins. This aspect of cystine utilization deserves close study.

The fact that methionine-S^{35} was not found in the carcasses of flies which had been fed either a sugar-skim milk diet or a complete synthetic amino acid mixture containing cystine-S^{35} indicated that methionine could not be synthesized from cystine by the fly. The results with a methionine-free diet show that the presence of methionine in the previous test diets did not interfere with cystine utilization under these conditions.

Another possibility existed, that methionine was produced from cystine but excreted as soon as it was formed. An examination of the feces plus regurgitum showed no evidence for the presence of methionine, indicating that no methionine was produced under the experimental conditions.

The sulfate-S^{35} ion was not found in either the hydrolyzates, the extracts or the extracted residues of whole flies fed cystine-S^{35}. The feces and regurgitum were examined to determine whether $SO_4^=$ was excreted as fast as it was formed. Large amounts of $SO_4^=$ were found in the feces indicating that the $SO_4^=$ is produced, then excreted very rapidly indeed. Apparently, physiological balance does not permit retention of more than traces of this anion within the body of the house fly.

Labeled taurine was found in the mixture of feces and regurgitum. This

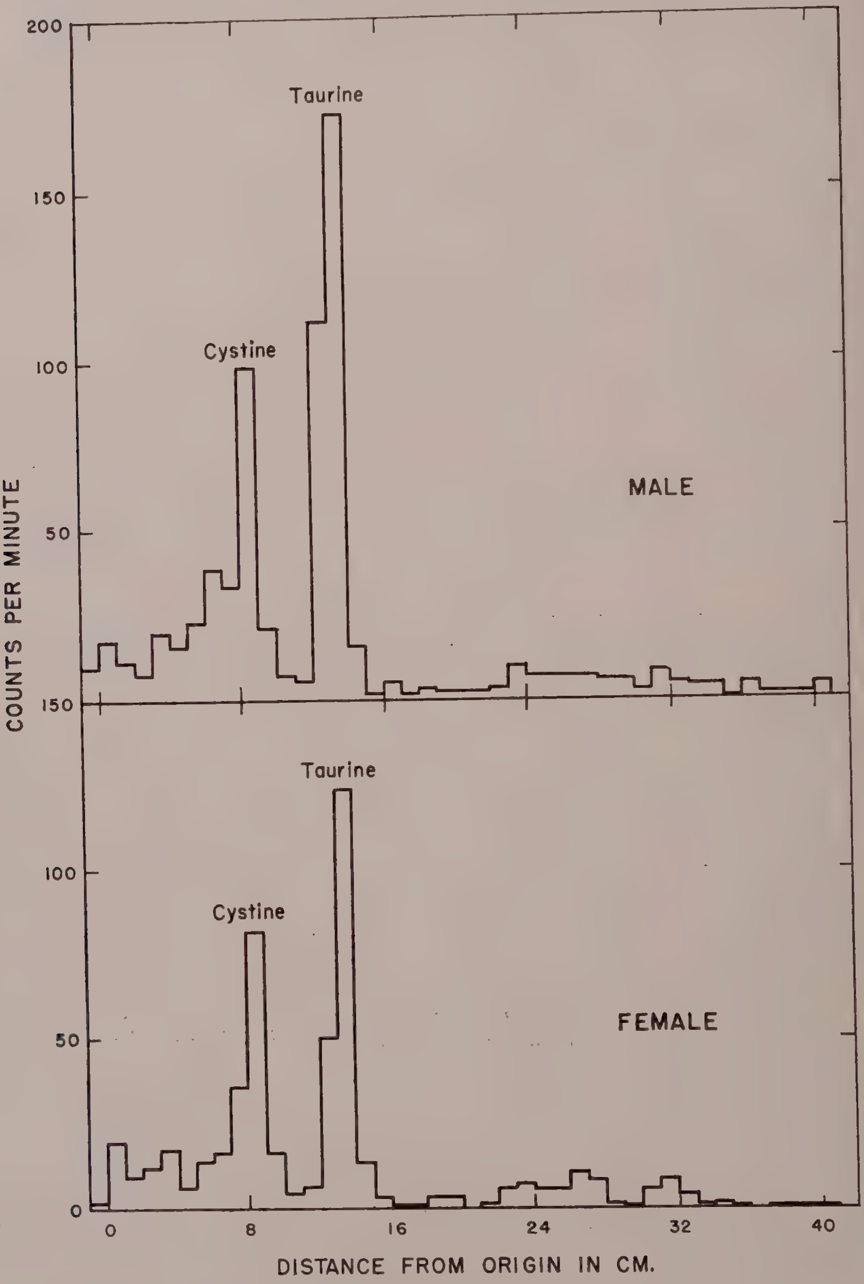

FIGURE 4. Location of radioactive substances on chromatograms of extracts of house flies fed a diet containing cystine-S^{35}.

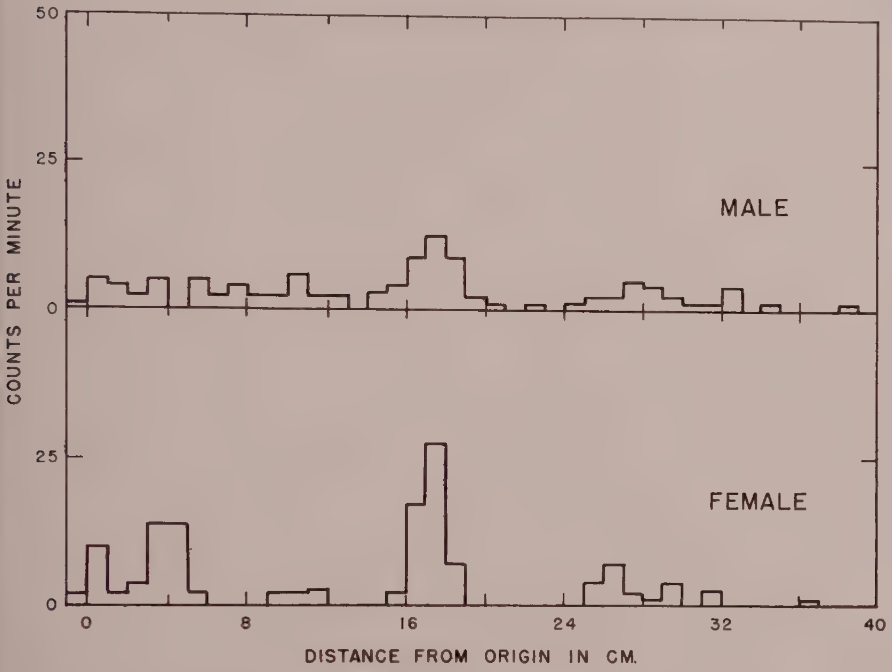

FIGURE 5. Location of radioactive substances on chromatograms of hydrolyzates of extracted residues from house flies fed a diet containing cystine-S^{35}.

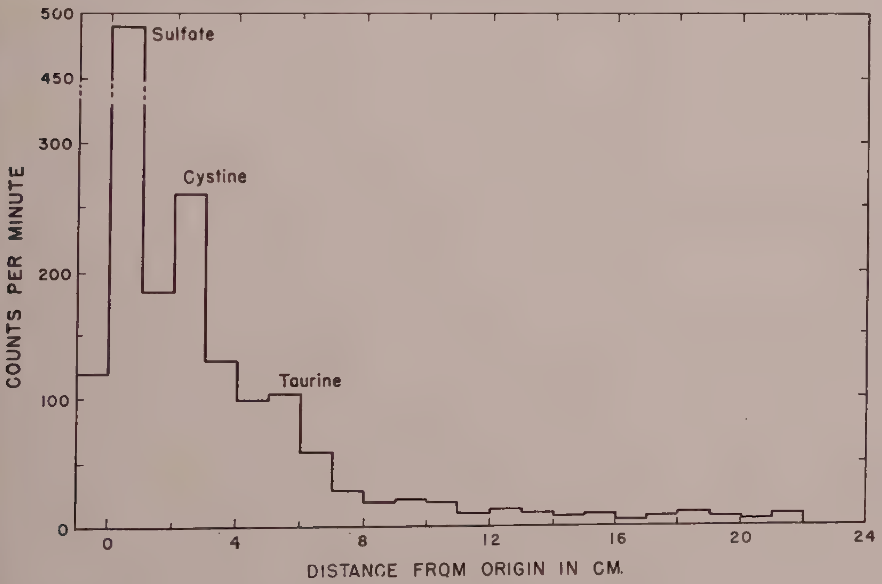

FIGURE 6. Location of radioactive substances on a chromatogram of feces from house flies fed cystine-S^{35}.

TABLE I

Radioactivity of Metabolites Isolated by Isotopic Dilution from Musca domestica L. Fed an Amino Acid Mixture Containing Cystine-S^{35} and Methionine

Sex	Crystallization order*	Specific activity in cpm./mg. of isolated metabolite			
		Cystine	Methionine	Taurine	Sulfate
Male	1st	47	4	465	26
	2nd	62	5	298	9
	3rd	53	4	318	0
Female	1st	65	7	294	—
	2nd	84	4	308	—
	3rd	75	5	273	—
	4th	67	3	—	—

* Recrystallizations were discontinued when specific activity became relatively constant or nil, or when the supply of isolate was exhausted.

TABLE II

Radioactivity of Metabolites Isolated by Isotopic Dilution from Musca domestica L. Fed an Amino Acid Mixture Containing Cystine-S^{35} but No Methionine

Sex	Crystallization order*	Specific activity in cpm./mg. of isolated metabolite			
		Cystine	Methionine	Taurine	Sulfate
Male	1st	37	8	735	5
	2nd	23	6	820	0
	3rd	24	3	712	—
	4th	25	4	783	—
Female	1st	43	10	239	8
	2nd	34	5	287	0
	3rd	16	3	223	—
	4th	13	—	267	—

* See footnote, Table I.

TABLE III

Radioactivity of Metabolites Isolated by Isotopic Dilution from Feces of Musca domestica L. Fed Cystine-S^{35} in the Absence of Methionine

Crystallization order*	Specific activity in cpm./mg. of isolated metabolite		
	Cystine	Taurine	Sulfate
1st	42	66	322
2nd	46	39	337
3rd	31	30	389
4th	32	30	—

* See footnote, Table I.

indicates that organic sulfur need not be degraded completely to $SO_4^=$ before leaving the fly's body but can be excreted in the form of taurine. Labeled cystine was also found in the feces, but it is not known whether this material was excreted, regurgitated from the crop, or simply moved through the digestive tract unabsorbed. Injection tests with labeled cystine must be carried out to resolve this question.

It is remotely possible that the microflora and microfauna of the gut were responsible for the production of taurine and/or $SO_4^=$ found in these experiments. However, this hardly seems likely; for if contaminating commensals capable of producing taurine and $SO_4^=$ were present, the likelihood is very great indeed that commensals capable of synthesizing methionine were also present. Since the activity of methionine producers was nil, the activity of taurine or $SO_4^=$ producers must in all probability also be nil under these experimental conditions. Nevertheless, this hypothesis will need to be verified by aseptic tests.

TABLE IV

PEAK RADIOACTIVITIES OF TAURINE AND CYSTINE ON CHROMATOGRAMS FROM HYDROLYZATES OF MUSCA DOMESTICA L.

Diet in which cystine-S^{35} was fed	Peak height in cpm.				Total in cpm.	
	Cystine		Taurine			
	Male	Female	Male	Female	Male	Female
Skim milk	120* (24)	80	885* (177)	163	1005	243
Amino acid mix with methionine	86	129	285	205	371	334
Amino acid mix without methionine	247	185	932	410	1179	595

* Data obtained from a 2-μl. chromatogram (given in parentheses) were multiplied by five for comparison with all other data which were based on 10-μl. chromatograms.

The absolute radioactivity of chromatograms of the same metabolites varied when the chromatograms were derived from hydrolyzates of insects fed different diets (Table IV). All of the data derived from whole carcasses of insects are based on a constant dry weight of starting material. While this source of variation has thus been ruled out, other small manipulatory errors on the order of ± 10 per cent occur inherently in hydrolysis and chromatography. These do not, however, account for the large differences observed in the results. Such effects cannot be explained at present, but might be due to such factors as differences in physiological state of the test insects; that is, larval history, degree of inanition and preoccupation with other biological activities such as reproduction. On the other hand, the

TABLE V

RATIOS OF THE SULFUR^{35} RADIOACTIVITY OF THE TAURINE AND CYSTINE PEAKS ON CHROMATOGRAMS OF HYDROLYZATES OF MUSCA DOMESTICA L. FED CYSTINE-S^{35}

Diet in which cystine-S^{35} was fed	Ratio of taurine activity to cystine activity	
	In males	In females
Skim milk	7.4*	2.0
Amino acid mix with methionine	3.3	1.6
Amino acid mix without methionine	2.8	2.2

* Data obtained from a 2-μl. chromatogram (given in parentheses) multiplied by five for comparison with all other data which were based on 10-μl. chromatograms.

question arises as to whether specific variations made in the composition of the diet could have affected the amounts of cystine converted to taurine and $SO_4^=$ as well as the amounts of these three substances retained in the bodies of the flies. Since the experiments are not so comprehensive as to answer this question, further study must be carried out before a valid answer can be made.

Despite these variations, certain generalizations can be drawn. The data given in Table V show the ratios of the activities of taurine and cystine on chromatograms in terms of the height of the peaks on the histogram. It is clear that the test insects of both sexes always contained more S^{35} in the form of taurine than in the form of cystine. The data in Table VI show that males always contained distinctly more taurine than did females. Moreover, in two cases out of three the cystine-S^{35} content in the male flies was greater than that in the females. In the third case males contained only slightly less cystine-S^{35} than did the females. Finally, these data show that in all cases the males contained more total S^{35} than did the females.

TABLE VI

SEXUAL DIFFERENCES IN THE ACTIVITY OF SPECIFIC METABOLITES OF CYSTINE-S^{35} FOUND IN MUSCA DOMESTICA L.

Diet in which cystine-S^{35} was fed	Ratio of activity found in male to activity found in female		
	Taurine	Cystine	Cystine and taurine
Skim milk	5.4*	1.5*	4.1*
Amino acid mix with methionine	1.4	0.7	1.1
Amino acid mix without methionine	2.3	1.3	2.0

* Data obtained from a 2-μl. chromatogram multiplied by five for comparison with all other data which were based on a 10-μl. chromatogram.

These results pose two important questions. First, what are the metabolic and biological roles of taurine; is it simply a convenient biochemical route for the removal of excess sulfur present originally as cystine? Is there a specific biological function which taurine serves in such major capacity as to require the synthesis of massive amounts of this substance? The second question concerns the factors responsible for the differences in cystine-S^{35} intake displayed by the two sexes of flies. That is, is this phenomenon directly related to specific sexual activities or indirectly related to more basic physiological phenomena? For instance, if male flies are more active than females, their water requirement might be increased. If so, the males' consumption of cystine-S^{35} solution would be greater; thus a remote effect on total S^{35} intake might be had. Clearly, further research is necessary to complete the understanding of even the qualitative aspects of cystine metabolism in the house fly.

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PHYSIOLOGICAL AND MORPHOLOGICAL EFFECTS OF GIBBERELLIC ACID ON EPICOTYL DORMANCY OF TREE PEONY

Lela V. Barton and Clyde Chandler[1]

SUMMARY

Gibberellic acid has been found to promote the growth of dormant epicotyls of tree peony (*Paeonia suffruticosa* Haw.) replacing the need for low temperature pretreatment for the production of green shoots in the greenhouse. Application of 1, 10 or 100 μg. to the hypocotyl of the germinated seed before planting in soil caused the emergence of green shoots above ground in three weeks or less. Most rapid development of the epicotyl followed treatment with 100 μg. gibberellic acid, but more normal growth resulted from the lower dosages. This is the only chemical which has been found to break epicotyl dormancy of tree peony.

Morphological studies have shown that differentiation of epicotyledonary tissues and elongation of the epicotyledonary shoot proceeds slowly in untreated seedlings in soil in the greenhouse. After application of gibberellic acid, however, both differentiation and elongation were rapid as shown by microscopic examination of sectioned material. Elongation of the epicotyl induced by gibberellic acid or by a period of 8 weeks at 5° C. was characteristic of seedlings which were capable of green shoot production in soil in the greenhouse. Such elongation did not occur in untreated seedlings which failed to produce green shoots. Elongation of the petiole is due to an increase in cell size and cell number.

INTRODUCTION

Tree peony (*Paeonia suffruticosa* Haw.) seeds if placed in a moist medium at temperatures of about 25° C. will germinate and form roots. However, if the germinated seedlings are left at this high temperature, green shoots rarely appear above ground in spite of the extensive root growth made possible by the large amount of stored food in the seed. This means, then, that the root is not dormant, but the portion of the seedling which forms the shoot is dormant. This condition has been called epicotyl dormancy, which can be broken by subjecting the seedlings, with the roots already growing, to a temperature of 1° to 10° C. for a period of 2 to 3 months (1). At temperatures as high as 15° C. a few seedlings will appear above ground.

From time to time since the discovery of epicotyl dormancy, unsuccessful attempts have been made to overcome it by using chemicals, especially those reported to break other types of bud dormancy. Among the chemicals

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tried in this laboratory have been indoleacetic acid, kinetin, thiourea, ethylene chlorohydrin, and ethylenediaminetetraacetic acid.

Since gibberellic acid has been found to promote the growth of physiologic dwarfs produced from nonafter-ripened embryos of *Malus Arnoldiana* Sarg., a response known to be brought about by low temperature (2), this chemical has been used in the present tests to break epicotyl dormancy of tree peony. The latter is similar to the physiologic dwarfs of *Malus* in that the growth of the green shoot of the plant is affected in both cases, though the untreated shoot does not appear above ground in the case of the tree peony.

Physiological and morphological responses of tree peony seedlings to both low temperature and gibberellic acid treatments will be described.

GROWTH RESPONSES

MATERIALS AND METHODS

Tree peony seeds of the 1956 crop, collected at Yonkers, N. Y., were mixed with moist granulated peat moss and held at a daily alternating temperature of 15° to 30° C. (16 hours at 15° and 8 hours at 30° C. daily for five days and at 15° C. for two days each week). Under these conditions roots began to be produced in about 6 weeks, and continued for a total of 3 months with a peak of germination in 8 or 10 weeks. Seedlings were usually removed from the peat moss when the roots were 20 to 30 mm. long, though some of the tests were made with longer roots. The appearance of the seedlings at this stage is to be seen in Figure 1 A. After removal from the peat moss, the seedlings were planted in 6-inch pots in a mixture of sod soil, granulated peat moss and sand in equal parts. Fifteen to 25 seedlings were planted in each pot.

Different methods of applying the gibberellic acid (GA) were tried. Soaking in water or in the acid solutions resulted in injury to the seedlings. Application of GA in lanolin was effective, but the dosage was difficult to determine. In all the experiments reported below, a known dose was applied with a micropipette to the hypocotyl of each seedling at the point of emergence from the seed (see Fig. 1 A). Seedlings which were to be treated with GA were planted so that the seed itself and the first few millimeters of the germinating hypocotyl remained above the soil. Not more than 10 μl. of solution could be applied at a time, since some of it might be lost. Consequently, for the higher dosages, several applications were made on the same hypocotyl, allowing time for drying between applications. Excessive drying of the exposed seedlings during any one operation was prevented by inverting half of a Petri dish over the pot while the solutions dried. The soil was watered thoroughly before the seedlings were planted. After treatment and drying of the hypocotyls, the seeds were covered with moist soil, but no water was added to the top of the pot until the next day. This permitted the absorption of the chemical without undue dilution.

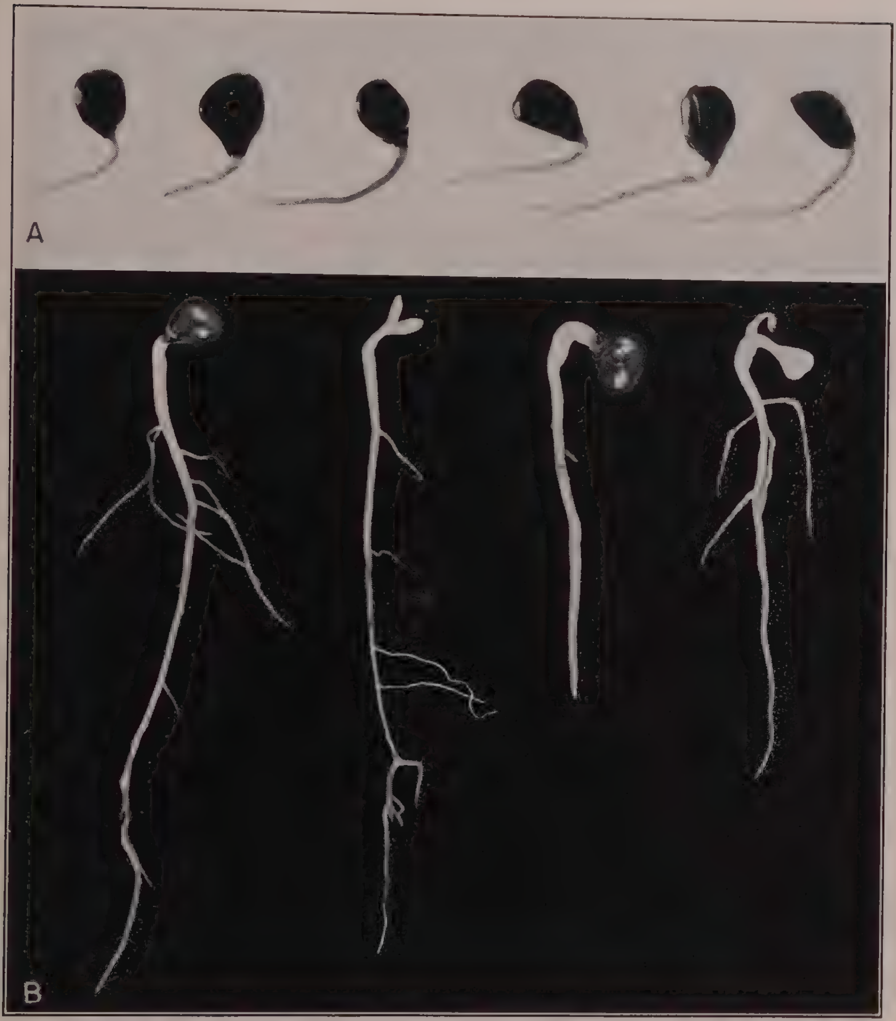

FIGURE 1. Tree peony. (A) Roots produced in moist granulated peat moss at a daily alternation of 15° to 30° C. showing stages at which seedlings may be treated for the breaking of epicotyl dormancy. (×0.95). (B) Seedlings 8 weeks after planting in soil. Two seedlings from greenhouse (left) and two from 5° C. (right). In each case one seedling has been removed from enveloping coats and endosperm (×0.83).

The pots were placed in the greenhouse for green shoot production, with and without a preceding period in a room at 5° C.

RESULTS

It was found very early that GA would replace the cold requirement for the growth of the epicotyl to form green shoots in the tree peony. Application of as little as 1 μg. per seedling caused some of the shoots to grow in the greenhouse without any low temperature pretreatment. Ten μg. were more effective, while 100 μg. provided the quickest response. Green shoot production after seedling treatment with 100 μg. of GA is shown in Table I. It will be seen that 97 per cent of the germinated seeds treated with GA formed green shoots without low temperature pretreatment, while only 5 per cent of those not receiving GA produced green shoots under these conditions. The latter grew very slowly as shown in Figure 2 A, while seedlings treated with 10 or 100 μg. of GA grew rapidly.

TABLE I

EFFECT OF LOW TEMPERATURE AND GIBBERELLIC ACID APPLICATION ON THE DEVELOPMENT OF GREEN SHOOTS IN SOIL IN THE GREENHOUSE. THREE REPLICATES OF 20 SEEDLINGS (ROOTS 20 TO 30 MM. LONG) PER TREATMENT

Weeks at 5° C.	Per cent shoot production after treatment with GA	
	None	100 μg./seedling
0	5	97
1	0	93
2	5	98
4	13	93
6	7	100
7	40	90

The effect of different periods at 5° C. preceding transfer to the greenhouse on subsequent green shoot production is shown in Table I for untreated seedlings and for those receiving 100 μg. of GA. Seven weeks at this temperature is not long enough to break the dormancy of all of the seedlings, but resulted in 40 per cent shoot production in the greenhouse. No low temperature was necessary for shoot production when the seedlings were treated with GA, but GA did not cause early emergence at 5° C. However, GA-treated seedlings which were at 5° C. for 7 weeks after treatment before removing to the greenhouse (Fig. 2 B) were sturdier than those which were produced in the greenhouse without a period at low temperature (Fig. 2 A). Also, it should be noted that the seedlings which had received only 10 μg. of GA appeared more normal than those treated with 100 μg. of GA. The latter tended to have smaller leaves of lighter green color and more spindly stem growth. GA treatment alone (10 or 100 μg.) without 5° C., produced even smaller leaves together with spindly growth (Fig. 2 A).

The length of the roots, and hence the development of the seedling, at the time they were removed from the granulated peat moss for planting and treatment determined to some extent the percentage as well as the speed of emergence after treatment. For example, germinated seeds with roots 40 to 50 mm. long when the hypocotyls were treated with 100 μg. of GA produced 10 per cent green shoot emergence in the greenhouse 8 days

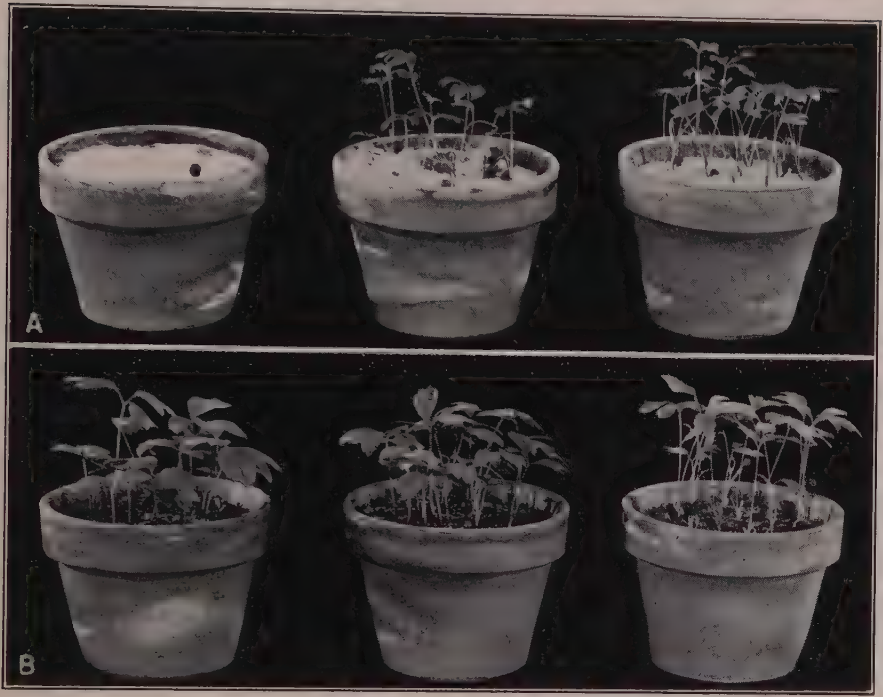

FIGURE 2. Green shoot production from tree peony seedlings planted in soil (20 seedlings per pot). (A) Greenhouse controls and (B) pretreated at 5° C. for 7 weeks before transferring to the greenhouse. Left to right: no gibberellic acid, 10 and 100 µg. GA at the time of planting in soil.

after planting, 50 per cent after 21 days and 80 per cent after 28 days. When the roots were only 20 to 30 mm. long at the time of treatment, initial shoot emergence was delayed to 18 to 21 days. When 10 µg. of GA were used, initial shoot emergence above soil took place in about 21 days

TABLE II

EFFECT OF SEEDLING SIZE AT TIME OF PLANTING ON THE RESPONSE TO LOW TEMPERATURE PRETREATMENT AND GIBBERELLIC ACID APPLICATION. TWENTY SEEDLINGS PER TREATMENT

Weeks at 5° C.	Root length, mm.	Per cent shoot production after treatment with GA		
		0 µg.	10 µg.	100 µg.
0	40–50	5	80	85
	20–30	5	55	85
7	40–50	85	95	100
	20–30	40	95	90

regardless of root length at the time of treatment. Seedlings with roots 40 to 50 mm. long planted in soil and left at 5° C. for 7 weeks gave 85 per cent seedling production in the greenhouse as compared with 40 per cent for roots which were 20 to 30 mm. long when placed at 5° C. (Table II), though the rate of emergence was about the same.

MORPHOLOGICAL STUDIES

MATERIALS AND METHODS

Morphological studies were made of the tree peony seedlings at different stages of development of the epicotyl. Comparisons were made of the changes which take place in the greenhouse without green shoot production and those which accompany production of green shoots after low-temperature or GA treatment.

Material for these studies was secured in the general manner described above for GROWTH RESPONSES. Fifteen germinated seeds which had been held in moist granulated peat moss at 15° to 30° C. daily alternation until the roots were 15 to 25 mm. in length were fixed and studied without any further treatment. At the same time, fifteen seeds each were planted in pots which were placed in the greenhouse or at 5° C. for periods of 1, 2, 3, 4, 6 and 8 weeks. Also, fifteen germinated seeds each were planted in pots after treatment of each hypocotyl with 0, 1, 10 or 100 μg. of GA, one pot of each to be examined 2, 4, 7, 9, 11, 14 and 28 days after planting in soil. These latter pots were all placed in the greenhouse. Duplicate pots were planted in all cases; one for morphological examination and one for observation on subsequent growth response.

Freehand sections were made in the epicotyl region of five seedlings at each examination period. The other ten embryos from the pot were dissected and the epicotyledonary portions were prepared for microscopic examination by fixing in formalin-propiono-alcohol (3, pp. 41–42); dehydrating in *tertiary* butanyl alcohol; imbedding in paraffin (Fischer's Tissuemat, m.p. 52°–54° C.); sectioning longitudinally at 20 μ; and staining with safranin (2.0 g. safranin per 100 ml. of 50 per cent ethanol) followed by Fast Green (0.2 g. per 100 ml. of 95 per cent ethanol).

The different regions of the epicotyl were measured with an ocular micrometer.

The primary roots of the 15 seedlings from each treatment were measured when it became evident that differences in length were significant. Observations on secondary roots consisted of a count of the total number and a measurement of the longest one on each seedling.

RESULTS

Epicotyl development in seedlings without GA treatment. The epicotyl in seeds which had been held in moist granulated peat moss at 15° to 30° C.

for two months, and which had germinated to form roots 15 to 25 mm. long, showed very little, if any, differentiation of tissues (Fig. 3 A). Compared with dry seeds, the epicotyl had undergone some cell division and the growth was somewhat extended but no differentiation of tissue into leaf primordia was observed. One week after seeds were planted in soil and placed in the greenhouse, the epicotyl began to elongate slightly and the initiation of the first leaf was evident (Fig. 3 B). Two weeks after planting, the first leaf doubled in length and the stem tissue was well differentiated in some cases (Fig. 3 C), while it remained less differentiated in others. During the next four weeks, growth and differentiation proceeded slowly and after the eighth week, the entire epicotyl was only 1.5 mm. in length (Fig. 3 D).

During the first six weeks after planting in soil, the epicotyl in seedlings placed at 5° C. developed more slowly than those placed in the greenhouse. Four weeks at 5° C. were required for the epicotyl to reach the stage of differentiation that was obtained in the greenhouse in two weeks. Furthermore, the first leaf was only two-thirds as long at 5° C. as in the greenhouse (Fig. 3 E). After six weeks at 5° C., growth and differentiation of the epicotyl (Fig. 3 F) was comparable to that obtained after four weeks in the greenhouse. However, the cotyledons and hypocotyl were greatly enlarged at 5° C., and the epicotyl itself reached 2.5 mm. in length (Fig. 3 G), a growth much greater than that obtained from seedlings held in the greenhouse for eight weeks (Fig. 3 D).

In spite of the apparent satisfactory development of the epicotyl in the greenhouse, no after-ripening took place there since no green shoots appeared above ground in the duplicate pots. Comparable seedlings held at 5° C. for 8 weeks produced 7 per cent green shoots upon transfer to the greenhouse.

A comparison of the external appearances of seedlings held in the greenhouse or at 5° C. for eight weeks can be seen in Figure 1 B. All of the development shown here took place underground. Note that the new shoot had already broken out of the enveloping structures in one of the seedlings from 5° C. and would soon have been above ground, in spite of the retarded root growth.

Epicotyl development in seedlings with GA treatment. Application of GA to the germinated seed definitely stimulated epicotyl growth.

At the time material was fixed for sectioning, the epicotyledonary portion of the embryo was removed and photographed for a comparison of the stages of development. Two days after treatment no effect of the GA was observed, but after 9 days marked elongation of the epicotyl treated with GA was evident. At this time the epicotyl of untreated seedlings showed added differentiation but little or no elongation (Fig. 4 A), while seedlings treated with 1, 10 or 100 μg. of GA all showed elongation of the epicotyl

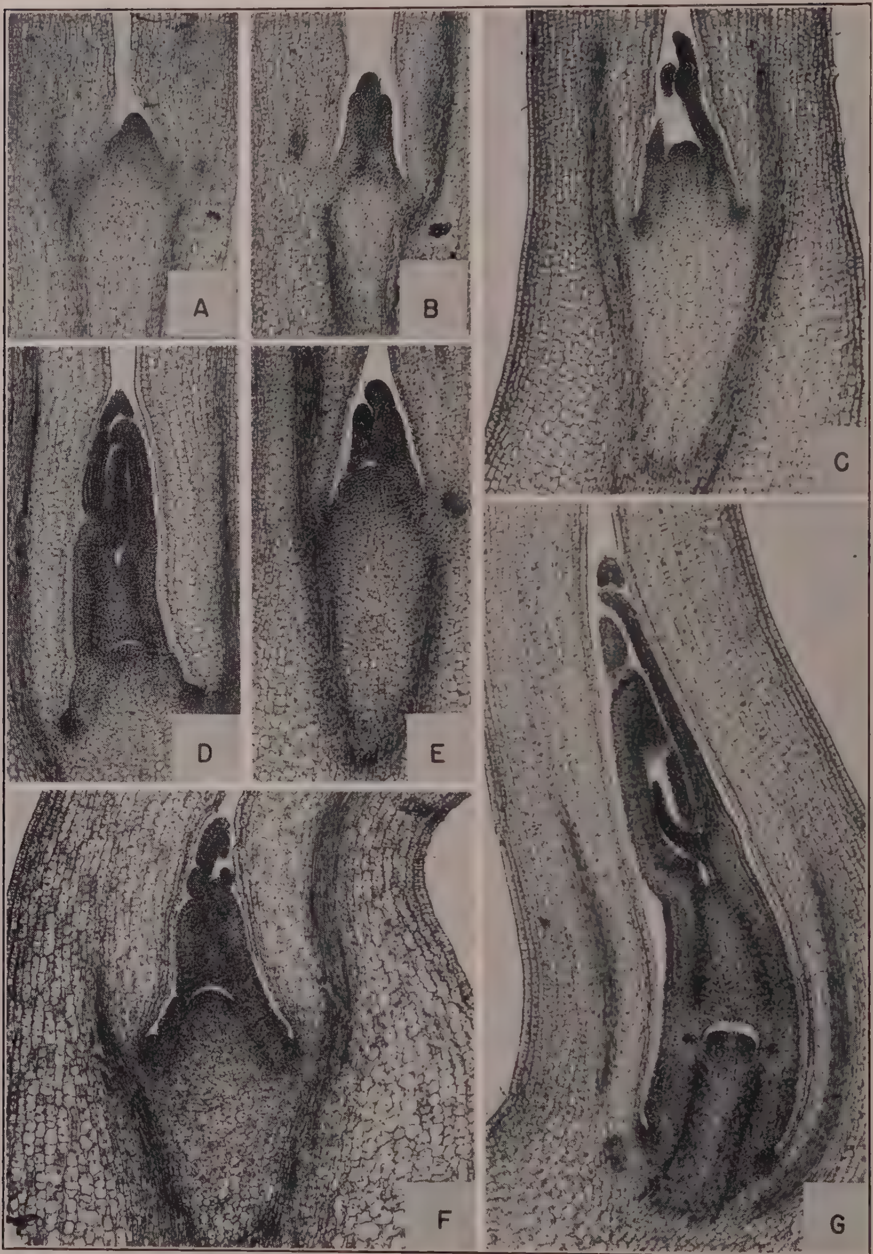

FIGURE 3. Sectioned epicotyls of tree peonies planted in soil. (A) At time of planting; (B, C, D) After 1, 2 and 8 weeks in the greenhouse; (E, F, G) After 4, 6 and 8 weeks at 5° C. (×27.5).

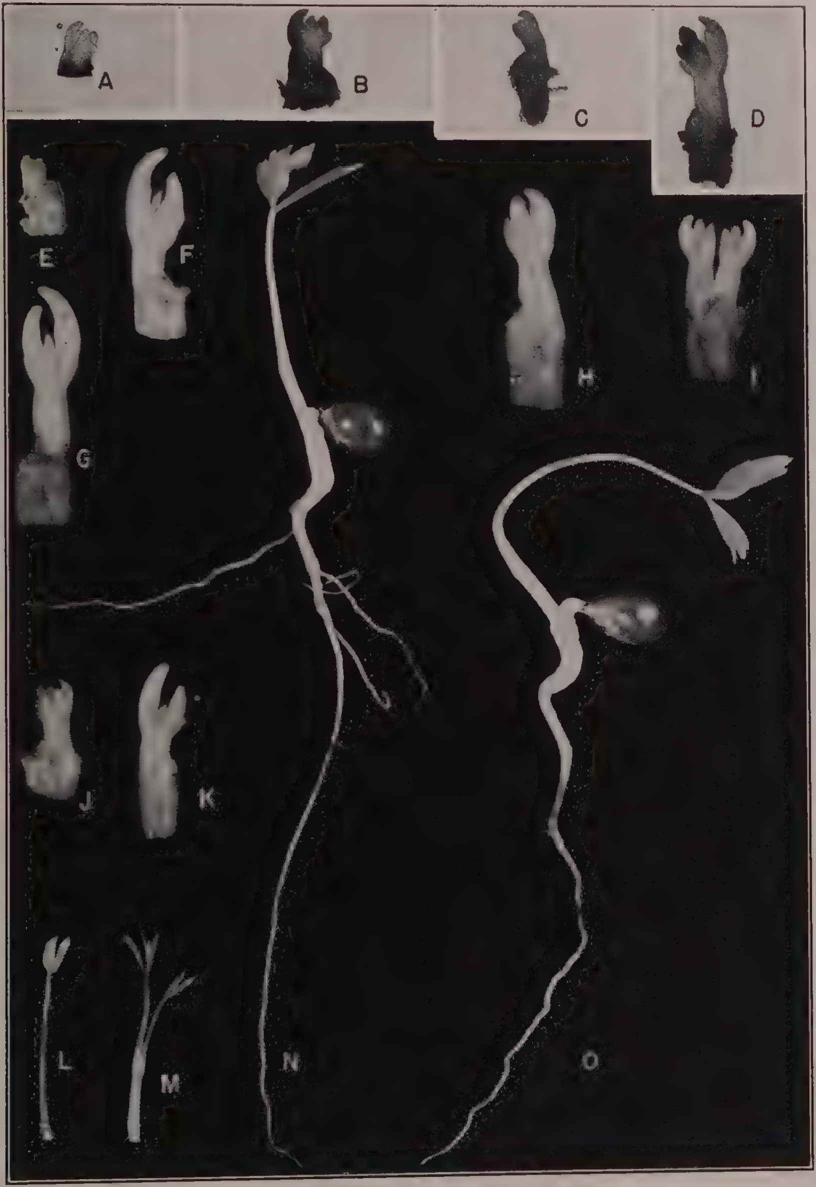

FIGURE 4. Development of the epicotyl of tree peony in soil in the greenhouse following treatment of the hypocotyl with gibberellic acid. Nine days after planting: (A) Untreated control; (B, C, D) Treated with 1, 10 and 100 μg. of GA. Fourteen days after planting: (E) Untreated control; (F, G, H) Treated with 1, 10 and 100 μg. of GA; (I) Twin epicotyl treated with 100 μg. of GA. Eighteen days after planting: (J) Untreated control; (K, L, M) Treated with 1, 10 and 100 μg. of GA. Twenty-eight days after planting: (N, O) Green shoots produced after treatment with 100 μg. of GA. (A-K, ×10; L-O, ×1).

(Fig. 4 B–D). One hundred μg. appeared most effective during the first few days after treatment. After fourteen days there was still no growth of the epicotyl in untreated seedlings (Fig. 4 E), but those in treated seedlings were more differentiated and approximately twice as long (Fig. 4 F–I). At this time 10 μg. of GA appeared more effective than 100 μg. Abnormal development was occasionally observed after treatment with 100 μg. of GA. This was the first indication of a possible abnormal growth response resulting from the larger dose, reflected, perhaps, in the extended stem length and smaller leaves seen in Figure 2 A.

After 18 days, the epicotyl in untreated seedlings began to elongate slightly (Fig. 4 J). No further development was noted for epicotyls from seedlings treated with 1 μg. of GA at this time (Fig. 4 K) but seedlings began to appear above ground in pots where they had been treated with 10 or 100 μg. of GA (Fig. 4 L, M). Twenty-eight days after planting, the latter seedlings were well established, with the first leaf well above ground (Fig. 4 N, O).

Studies of sectioned material also showed no effect of the GA two days after treatment. However, four days after treatment with 1 μg. of GA, the epicotyl was comparable to the development attained by untreated seedlings after two weeks in the greenhouse or four weeks at 5° C. Seven days after treatment with 1, 10 or 100 μg. of GA, epicotyl development was comparable to three weeks in the greenhouse. After nine days, differentiation was about the same as after 8 weeks in the greenhouse and was more advanced than after 6 weeks at 5° C. Greatest elongation of the epicotyl was obtained from 100 μg. of GA (Fig. 5 E), and 10 μg. were more effective than 1 μg. (Fig. 5 C, D). At this time the epicotyl in untreated seedlings was well differentiated (Fig. 5 B) but was only 0.65 mm. in length while epicotyls from 1, 10 or 100 μg.-GA treatments were 1.15, 1.63 and 1.90 mm. long. This was a slight increase in elongation but a marked advance in differentiation compared with development of the untreated epicotyl after four days (Fig. 5 A).

After 14 days, the most differentiation and elongation was obtained from 10 μg. of GA rather than from 100 μg. (Fig. 5 F), an effect noted above.

At the end of 28 days when the experiment was terminated, the maximum development of the epicotyledonary shoot in seedlings which had not appeared above ground was 1.1, 3.6, 4.8 and 3.1 mm. in untreated seedlings and in those which received 1, 10 and 100 μg. respectively (Fig. 6 A–D). At this time, duplicate pots held for growth responses had produced green shoots above ground to the extent of 0, 1, 40 and 59 per cent. The percentage emergence of these seedlings was somewhat lower than that shown in Tables I and II because of the smaller size of the seedlings (roots 15 to 25 mm. long) at the time of treatment.

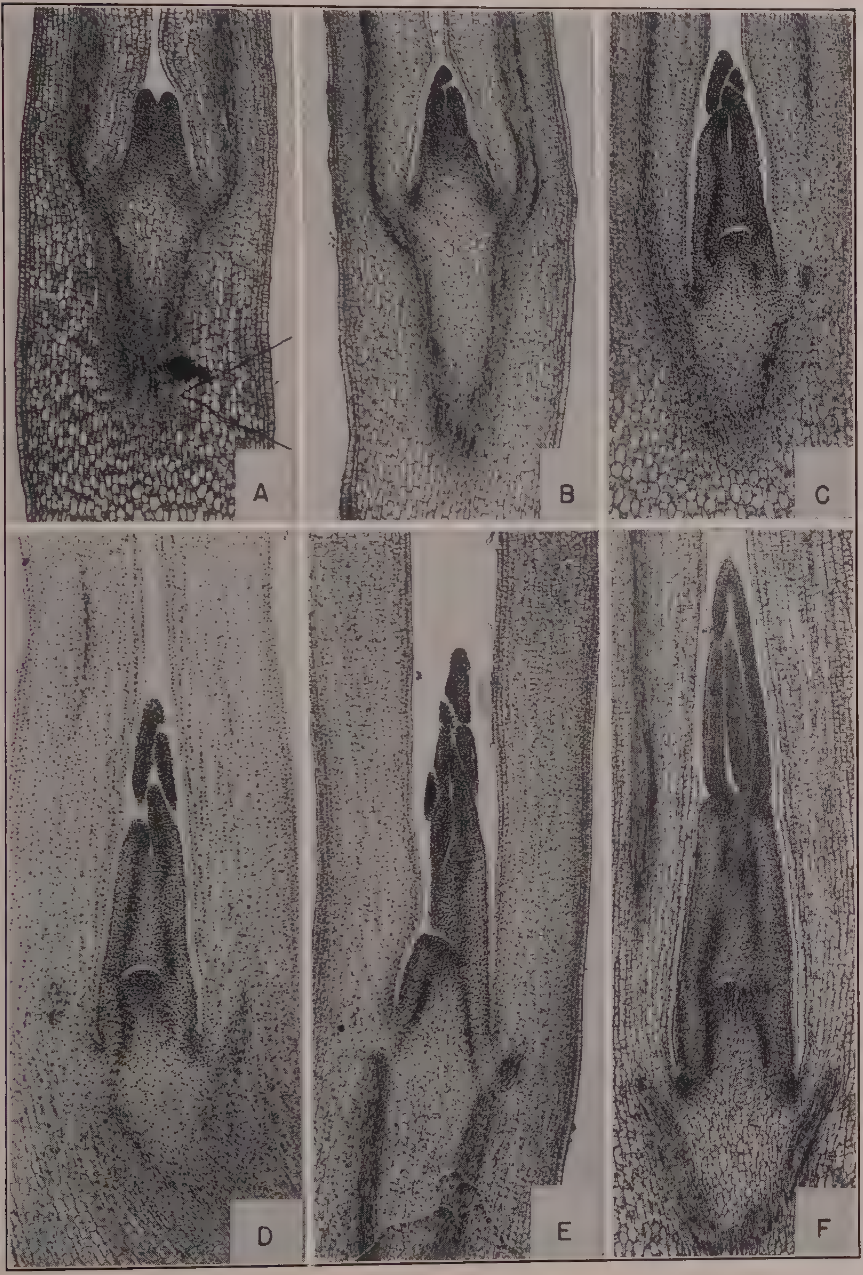

FIGURE 5. Sectioned epicotyls of tree peony seedlings planted in soil in the greenhouse following treatment of the hypocotyl with gibberellic acid. (A, B) Untreated control 4 and 9 days after planting; (C, D, E) Nine days after treatment with 1, 10 and 100 μg. of GA; (F) Fourteen days after treatment with 10 μg. of GA (×27.5).

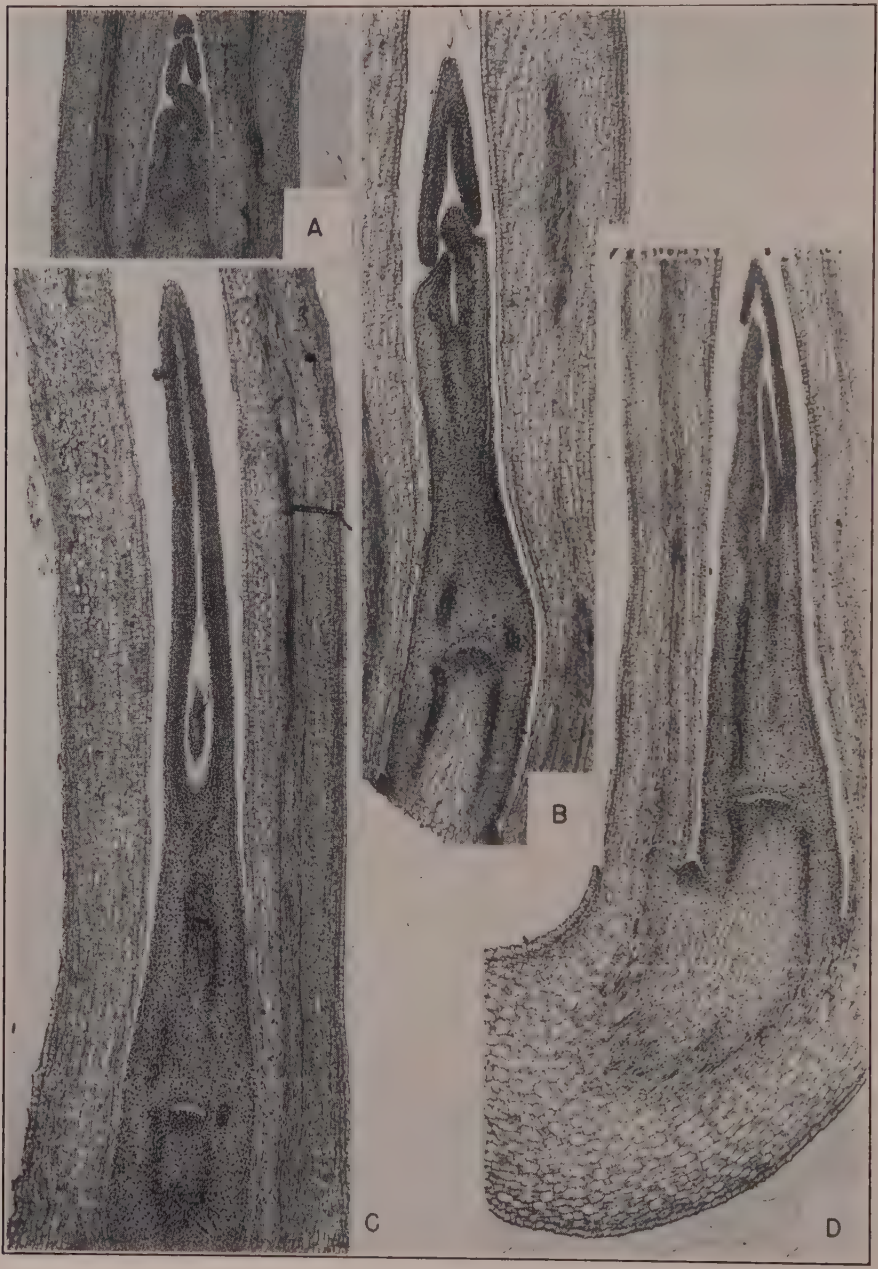

FIGURE 6. Sectioned epicotyls of tree peony seedlings 28 days after planting in soil in the greenhouse following treatment of the hypocotyl with gibberellic acid. (A) Untreated control; (B, C, D) Treated with 1, 10 and 100 μg. of GA (×27.5).

Since the length of the epicotyledonary shoot increased from 2.7 to 4.2 times that of the untreated seedlings as a result of GA application, measurements were made of the various regions of the epicotyl to locate the areas of greatest elongation.

Some of the data are shown in Table III. Four weeks after planting, the diameter of the epicotyl base (region 1) had increased after treatment with GA. In untreated material, the entire length of the epicotyl from its base to the first leaf (regions 2 and 3) was 0.59 mm., while in material treated with 1, 10 and 100 μg. GA, the corresponding lengths were 2.36, 2.31 and 1.61 mm. Region 4 showed marked elongation following GA treatment, measuring 1.28, 2.44 and 1.45 mm. after application of 1, 10 and 100 μg. as compared with 0.55 mm. for the untreated control. Total lengths of the epicotyledonary shoots after 4 weeks of growth in soil in the greenhouse were 1.14 mm. in untreated seedlings and 3.64, 4.75 and 3.06 mm. after treatment with 1, 10 and 100 μg. of GA. The decreased elongation after the highest dosage is again indicative of an excess of the chemical. The elongation of the GA-treated seedlings was accompanied by differentiation which was not to be found in untreated seedlings of the same age as shown above.

TABLE III

Regions of Elongation in the Epicotyledonary Shoot

Temp.	Treatment, GA μg./plant	Weeks after planting	Regions* in mm.				
			1	2	3	4	Total length
Greenhouse	0	4	0.36	0.59†	—	0.55	1.14
	1	4	0.57	0.86	1.50	1.28	3.64
	10	4	0.67	0.71	1.60	2.44	4.75
	100	4	0.67	0.48	1.13	1.45	3.06
Greenhouse	0	8	0.67	0.29	0.40	0.80	1.53
5° C.	0	8	0.65	0.56	0.86	1.48	2.90

* 1. Diameter of epicotyl at region of cotyledon attachment.
2. Longitudinal axis from region 1 to shoot apex.
3. Length of peticle above shoot apex.
4. Lamina above petiole.

† Combined length of regions 2 and 3.

After eight weeks in soil in the greenhouse the diameter of the epicotyl at the region of cotyledon attachment (region 1) had increased, but growth in length was still restricted (Table III). Elongation of the epicotyledonary shoot at 5° C. was greater than in untreated seedlings in the greenhouse but not as great as for GA-treated greenhouse seedlings.

Measurements were made of cells from the petiole (see Table III) of seedlings held in the greenhouse for 28 days. Average measurements for 50

TABLE IV

LENGTH OF THE PRIMARY ROOT AS A RESULT OF LOW TEMPERATURE OR GIBBERELLIC ACID TREATMENT OF SEEDLINGS

Temp.	μg. GA	Average root length in mm. after	
		2 wk.	4 wk.
Greenhouse	0	63	81
	1	72	117
	10	73	114
	100	68	86
5° C.	0	26	35

cells each of 17.8×14.9 microns for untreated seedlings and 23.6×16.0, 22.5×19.2 and 20.2×20.2 for those treated with 1, 10 and 100 μg. of GA respectively were obtained. Also, the numbers of cells in comparable lengths of this region were counted and found to be 14, 72, 78 and 51 respectively. It appears, then, that elongation is due to both increased cell size and cell number. Thinner sections of material will be required for more exact data on this point.

Morphological studies on rice, peas and *Vigna* have shown that cell elongation is, in the main, responsible for the extended growth of plants treated with gibberellins, though there have been some claims that cell division is also stimulated (5). Also, Sachs and Lang (4) have observed increased cell division in the subapical region of *Hyoscyamus niger*.

At the time material was fixed for morphological studies, measurements were made of the primary root length of each seedling. Average root lengths for 15 seedlings of each treatment are shown in Table IV. The lower concentrations of GA (1 and 10 μg.) stimulated root growth and 100 μg. gave no inhibition. There was little difference in the number of secondary roots produced but they were shorter on GA-treated than on untreated seedlings.

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THE EFFECT OF GIBBERELLIC ACID ON GERMINATION AND POLLEN-TUBE GROWTH

Clyde Chandler

SUMMARY

For a study of the effect of gibberellic acid (GA) on cell elongation, pollen was collected at random from twenty-seven plants representing sixteen different families and was cultured on a sucrose-agar medium. Gibberellic acid in concentrations of 31.25 to 1000 mg./l. was added to this medium. The percentage of germination and the rate of growth of the pollen tubes on the control and GA media were compared. Pollen from nine plants gave no germination on the control medium and was not stimulated by the addition of GA to the medium. Pollen from ten plants germinated on all media but tube growth was greatly inhibited by all concentrations of GA which caused coiling, enlarging of tips of tubes and even exuding of cytoplasm. Pollen from one plant which did not germinate on the control medium and would have been considered nonviable gave good germination in the GA medium. Pollen from seven plants showed an increase in the percentage of germination and a marked increase in tube length when GA was added to the medium.

INTRODUCTION

If gibberellic acid (GA) increases the rate as well as the amount of pollen-tube growth, it may be possible by applying the chemical to the stigma at the time of pollination to remove certain types of incompatibilities where the rate of pollen-tube growth prevents fertilization before the flowers abscise.

Various reports on the effect of GA on the extended growth of plants have attributed this effect to cell elongation (5). Since the pollen tube is a single cell which frequently does not attain sufficient growth to accomplish fertilization in certain self-incompatible plants, it seemed pertinent to study the effect of GA on pollen germination and pollen-tube growth.

Kato (4) found that gibberellic acid increased the growth of pollen tubes in *Lilium longiflorum*. In a concentration of 50 mg./l. where maximum growth occurred, the length of the tubes increased five times that of the control during five hours of incubation. However, he reported that tube elongation was inhibited with concentrations of 100 to 200 mg./l. of GA. Hayashi, according to Stowe (5), reported in 1940 that GA inhibited pollen-tube growth in cucumber.

MATERIALS AND METHODS

Flowers and large buds were collected at random from 27 plants representing 16 different families. These were brought into the laboratory and

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placed in small beakers filled with water, until the pollen was ready to be collected.

A medium of 1 gram of agar and 10 grams of sucrose dissolved in 100 ml. of tap water was used for all control tests. To 10 ml. of this medium, 10 mg. of GA were added while the medium was still warm. After mixing thoroughly, then cooling and reheating, 5 ml. of this medium were diluted by adding 5 ml. of the control medium. Four further dilutions were made in like manner, so that the final concentrations of GA used were 31.25, 62.5, 125, 250, 500 and 1000 mg./l.

Drops of media (0.2 ml.) were placed on glass microscope slides and pollen was distributed over the surface of each drop as soon as it solidified. Four sets of each series were made for observations at 2-, 4-, 6- and 24-hour intervals. All slides were placed in a moist chamber until time for observation, when one complete set of slides was removed and a drop of aceto-carmine was placed on each preparation. A cover glass completed the mount.

Fifty pollen tubes were measured on the control medium and on the GA medium giving the highest percentage germination with the longest tubes. All tubes were measured with an ocular micrometer and lengths were recorded in ocular units, which were later converted to microns. The percentage germination was obtained from counting at least 250 pollen grains and recording the numbers with and without tubes.

Gibberellic acid *ca.* 85 to 90 per cent pure was supplied through the courtesy of Eli Lilly and Co., Indianapolis, Indiana.

RESULTS AND DISCUSSION

Pollen germination. Pollen from nine plants: *Campanula* sp., *Chrysanthemum cinerariaefolium* Vis., *Eucharis grandiflora* Planch., *Gladiolus* sp., *Hibiscus Moscheutos* L., *Hibiscus syriacus* L., *Hosta plantaginea* Aschers., *Physostegia virginiana* Benth., *Salvia splendens* Sello cv. 'Blaze of Fire' did not germinate on the control medium and was not affected by the addition of GA to the medium. Different media were not tried.

Germination of pollen collected from freshly opened flowers of *Lilium speciosum* Thunb. cv. 'Rubrum' growing on the Institute grounds was definitely stimulated by GA (Table I, *Lilium* 1). After two hours none of the pollen grains had germinated on the control medium while 75 per cent of the grains on medium with 500 mg./l. of GA produced short tubes. After six hours, 37 per cent germination took place on the control with no increase in the number of tubes produced on the GA medium.

Pollen collected from the same *Lilium* species growing in a commercial greenhouse (Table I, *Lilium* 2) failed to germinate on the control medium even after 24 hours and, by conventional standards, would have been discarded as nonviable. On the medium with 500 mg. of GA, one-fourth of the grains germinated. Thus, gibberellic acid hastened and increased the

TABLE I

EFFECT OF GIBBERELLIC ACID ON POLLEN GERMINATION

Class	Hours of incubation	Control, per cent germ.	Gibberellic acid	
			Mg./l.	Per cent germ.
Lilium 1	2	0	500	75
Lilium 1	6	37	500	74
Lilium 2	24	0	500	24
Linaria	4	7	500	22
Lonicera	6	11	31.25	23
Petunia	2	24	500	23
Petunia	4	38	250	52
Petunia	6	29	250	47
Verbena 1	4	30	250	47
Verbena 2	4	22	125	47
Delphinium	6	75	500	75
Lobelia	2	87	250	92
Saintpaulia 1	6	76	500	67
Saintpaulia 2	6	60	500	48

germination of pollen of *Lilium speciosum* from two different sources. The difference in the concentration found most effective by Kato (4) and that reported in this paper may be due to the difference in plant material or to the solubility of the GA in the medium.

Germination of pollen of *Linaria vulgaris* Mill., *Lonicera japonica* Thunb., *Petunia hybrida* Vilm. cv. 'Snowstorm,' *Verbena hybrida* Voss cv. 'Spectrum Red,' and *Verbena hybrida* Voss cultivar was also stimulated by GA as seen in Table I, which shows the results with the optimum GA concentration and incubation time for each species. The best germination of *Lonicera* pollen was obtained with 31.25 mg./l. of GA while *Linaria*, *Petunia* and *Verbena* germinated better on media containing 125, 250 or 500 mg./l. The incubation time resulting in the highest germination was from 2 to 6 hours.

Pollen of *Delphinium* sp. and *Lobelia siphilitica* L. was not stimulated by the addition of GA to the medium in the concentrations shown in Table I or in any of the other concentrations used in these tests.

Pollen germination of both diploid and tetraploid *Saintpaulia ionantha* Wendl. cv. 'Amethyst' (*Saintpaulia* 1 and 2 in Table I) may have been somewhat inhibited by GA. Lower concentrations than those given in the table also showed this effect.

Pollen from the following seven plants: *Browallia americana* L. cv. 'Sapphire,' *Cleome spinosa* L., *Digitalis mariana* Boiss, *Digitalis lanata* Ehrk., *Rosa* cv. 'Betty Pryor' (Floribunda), *Torenia fournieri* Lind., and *Vinca rosea* L. germinated on the control medium, but on all media with GA either no germination was obtained or the few short tubes which were produced coiled or burst at their tips. Often pseudo-germination, or an extrusion of the contents of the grains, was obtained.

It is clearly evident that gibberellic acid may stimulate, inhibit or not affect pollen germination, depending on the species and, perhaps, on the physiological condition of the pollen at the time of testing.

Pollen-tube growth. Gibberellic acid not only increases the percentage of germination of certain pollen, but also speeds up the rate of growth of the tube as shown in Table II.

TABLE II

EFFECT OF GIBBERELLIC ACID ON POLLEN-TUBE GROWTH

Class	Hours of incubation	Control			Mg./l.	Gibberellic acid		
		Pollen-tube length (μ)				Pollen-tube length (μ)		
		Min.	Max.	Av.		Min.	Max.	Av.
Lilium 1	2	0	0	0	500	67	399	219
Lilium 1	6	48	219	96	500	124	1017	524
Lilium 2	24	0	0	0	500	67	618	301
Linaria	4	19	209	84	500	57	219	106
Lobelia	2	19	105	52	250	48	114	84
Lonicera	6	86	741	401	31.25	114	1824	805
Petunia	2	29	180	117	500	67	247	174
Petunia	4	57	304	166	250	67	371	243
Petunia	6	76	437	197	250	76	587	275
Verbena 1	4	95	827	491	250	238	1140	755
Verbena 2	4	171	1036	463	125	86	1131	642
Delphinium	6	86	684	294	500	48	304	138
Saintpaulia 1	6	76	323	186	500	10	67	25
Saintpaulia 2	6	29	513	258	500	10	105	32

Pollen of *Lilium* 1 (Table II and Fig. 1) produced tubes which measured 67 to 399 μ in length on medium containing 500 mg./l. of GA, while no tubes were produced on the control medium after two hours of culture. After six hours of incubation, tubes measured 48 to 219 μ (average 96) on the control medium and 124 to 1017 μ with GA (average 524). These figures indicate more than a fivefold increase in length as a result of adding GA to the medium. Tubes were long and straight except for a few enlargements where the cytoplasm appeared rather dense.

The lily pollen collected in the commercial greenhouse (*Lilium* 2, Table II) which would have been considered nonviable, since no germination was observed after 24 hours on the control medium, produced tubes 67 to 618 μ in length (with an average of 301 μ) on the medium with 500 mg./l. of GA. This was little more than one-half the length obtained from the pollen of *Lilium* 1 after six hours, but it does show that this pollen of low viability can be stimulated by GA.

Pollen-tube growth of *Linaria* was stimulated by GA (Fig. 2 A, B and Table II). Tubes on the control medium were short and somewhat coiled while tubes were straight and longer with 500 mg./l. of GA added to the medium.

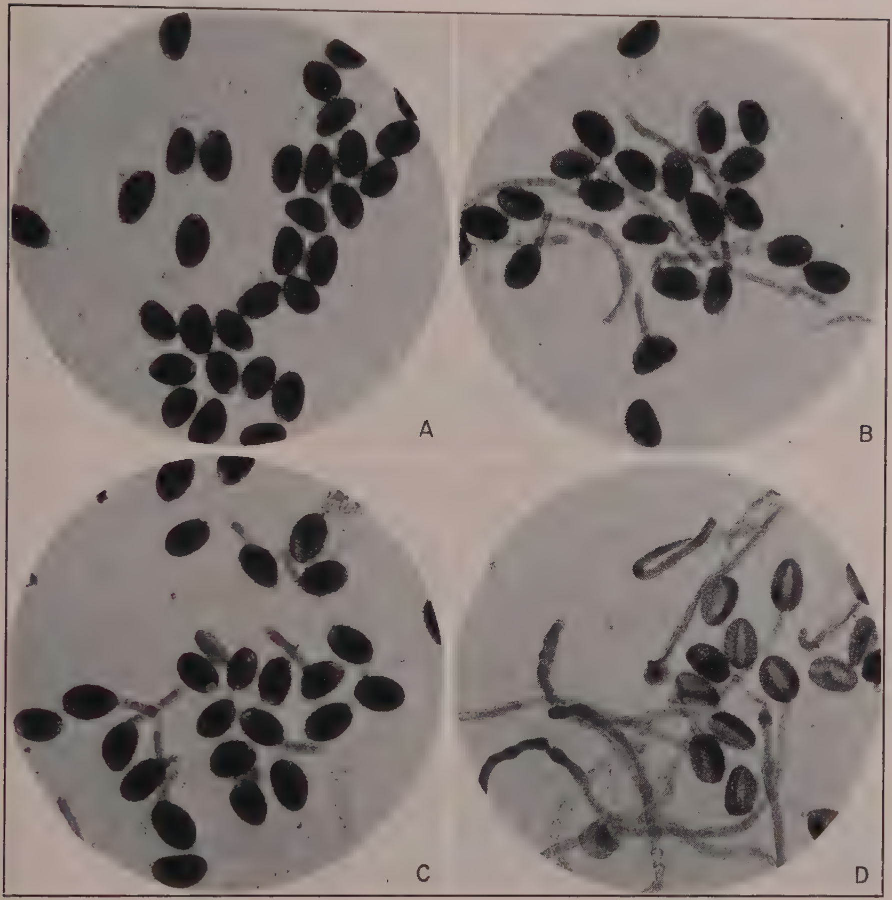

FIGURE 1. Pollen of *Lilium speciosum* cv. 'Rubrum.' After 2 hours of incubation: (A) Control; (B) 500 mg./l. of gibberellic acid (GA). After 6 hours: (C) Control; (D) 500 mg./l. of GA (×75).

Pollen tubes of *Lobelia*, *Lonicera* and *Petunia* were likewise stimulated by GA. Tubes of *Lobelia* reached their greatest length on the medium containing 250 mg./l. of GA with an average of 84 μ while tubes on the control medium averaged 52 μ in length. Two tubes from pollen of *Lonicera* were produced on medium containing 1000 mg./l. of GA. This was the only class of pollen which produced any tubes with such a high concentration of GA. The medium with GA at 31.25 mg./l., on which growth was twice that of the control, was most favorable for this species.

The stimulating effect of gibberellic acid on the growth of pollen tubes of *Petunia* is shown in Table II and Figures 2 C, D and 3. Two hundred fifty mg./l. of GA was most favorable for growth. During the first two

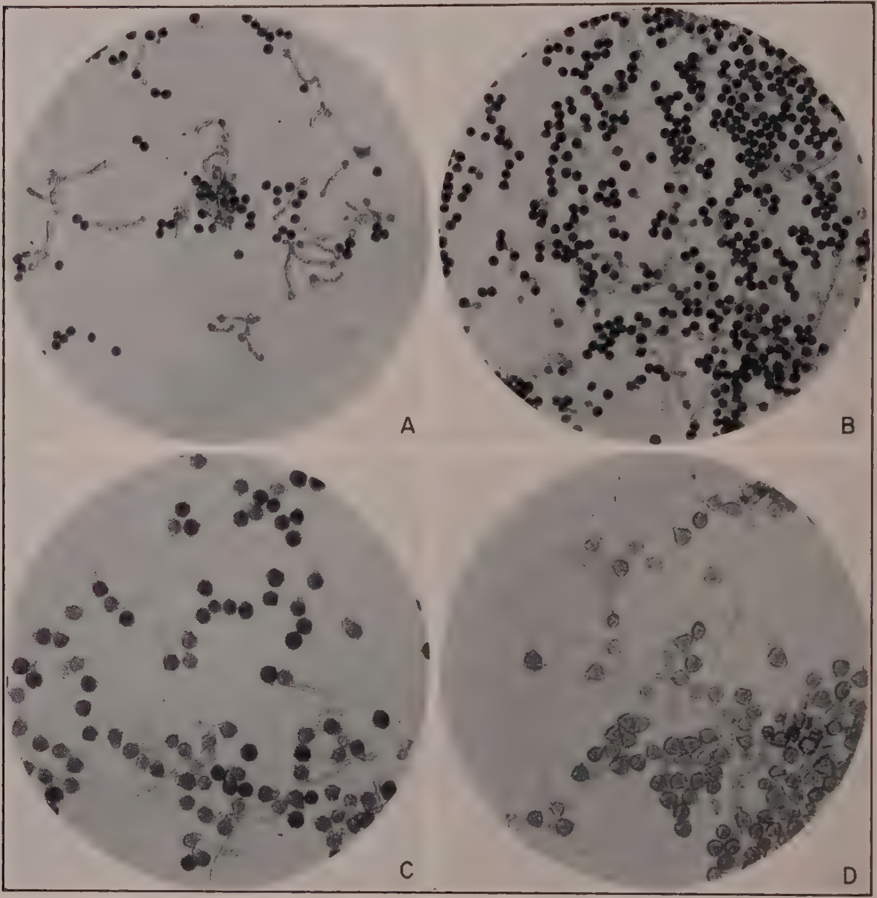

FIGURE 2. Stimulating effect of gibberellic acid (GA) on pollen-tube growth. *Linaria* pollen after 4 hours: (A) Control; (B) 500 mg./l. of GA. *Petunia* pollen after 2 hours: (C) Control; (D) 500 mg./l. of GA (×75).

hours of incubation, the tubes attained a greater length on the medium with 500 mg./l. of GA, but at the end of the four-hour period, these tubes were coiled and some had burst at their tips, so that continued growth was not possible. Some growth stimulation was noted at concentrations of GA weaker than 250 mg./l. but no growth took place at 1000 mg./l.

Pollen of *Verbena* 'Spectrum Red' (*Verbena* 1, Table II), showed an increase in rate of tube growth in the presence of 250 mg./l. of GA. The average rate of growth of this pollen on the seven different media used in this experiment is given in Figure 4. Pollen of the white *Verbena* (*Verbena* 2, Table II) grew best on a medium with 125 mg./l. of GA.

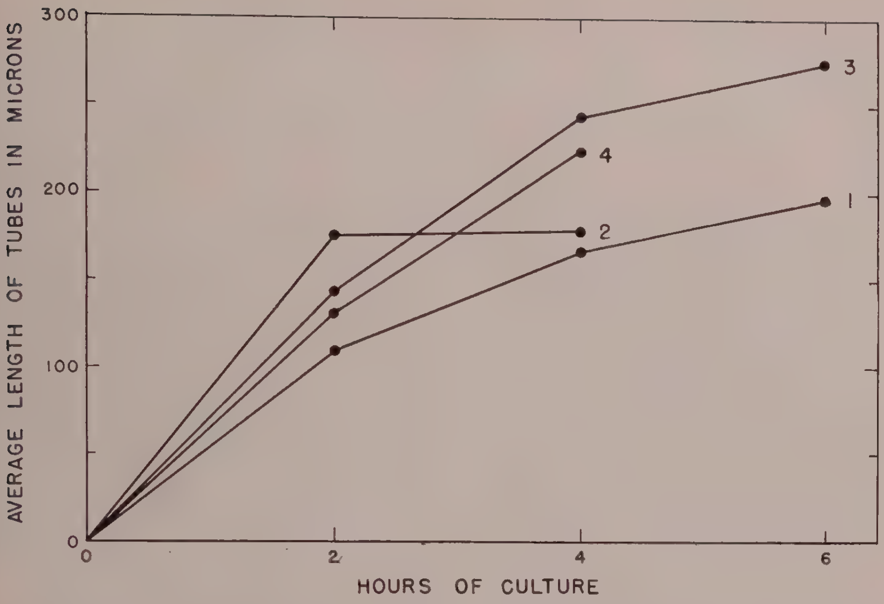

Figure 3. Stimulating effect of gibberellic acid (GA) on cell elongation as shown by pollen-tube growth in *Petunia* (after 2, 4 and 6 hours of incubation). 1. control medium, 2. 500 mg./l. of GA; 3. 250 mg./l. of GA; 4. 125 mg./l. of GA.

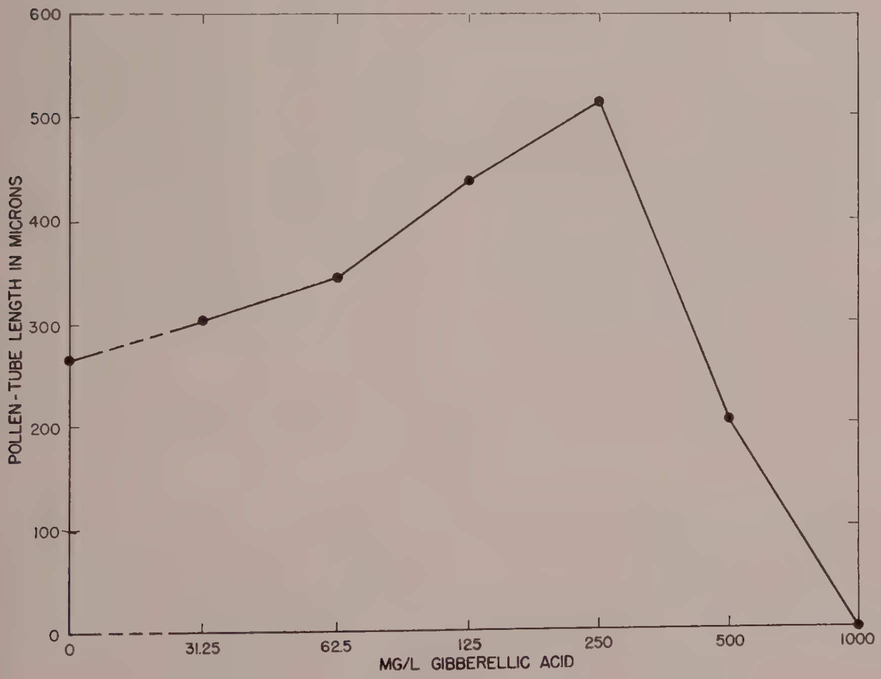

Figure 4. Pollen-tube growth of *Verbena* in the presence of gibberellic acid (GA).

Pollen tube growth of *Delphinium* was definitely inhibited by concentrations of GA included in this study. Tubes on the control medium were twice as long as those on a medium containing 500 mg./l. of GA, which gave the best growth of the GA media. Here, tubes were short, coiled and enlarged at their tips (Fig. 5 A, B). Pollen from both the diploid and tetraploid *Saintpaulia* (*Saintpaulia* 1 and 2, Table II) also produced short tubes in the presence of GA while long, straight tubes were obtained on the control medium. The inhibition of the tetraploid pollen is shown in Figure 5 C and D.

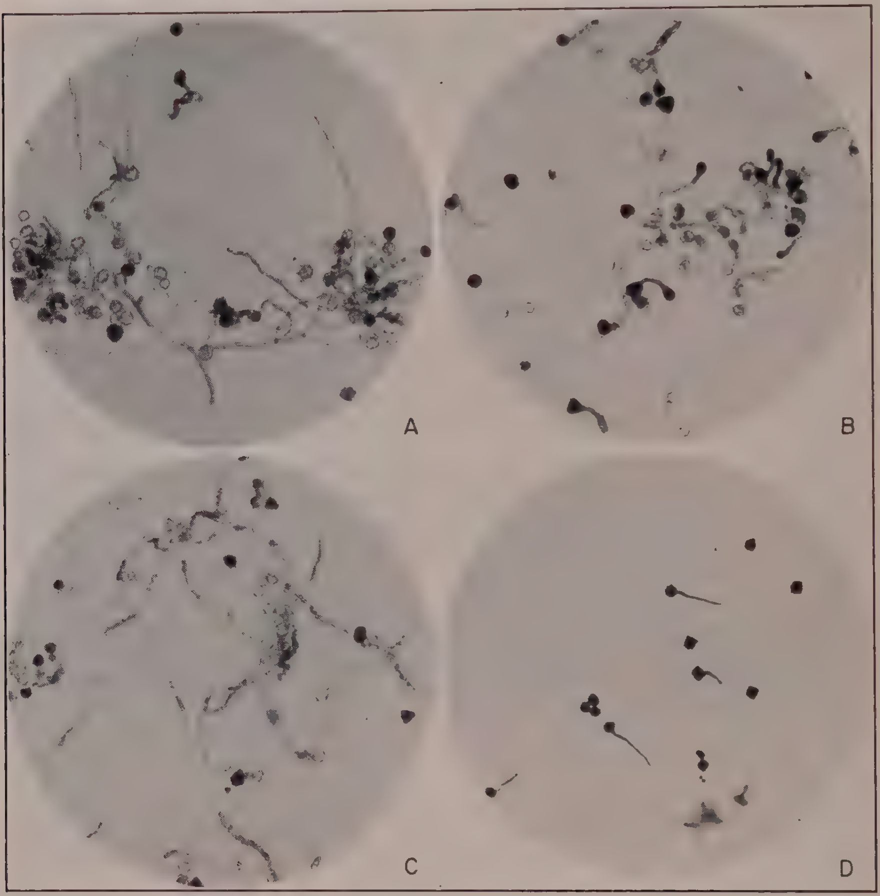

FIGURE 5. Inhibition of pollen-tube growth after 6 hours. *Delphinium*. (A) Control; (B) 500 mg./l. of gibberellic acid (GA). *Saintpaulia* (tetraploid). (C) Control; (D) 500 mg./l. of GA. (×75).

CONCLUSIONS

It is recognized that pollen is very sensitive to environmental conditions and that certain types of pollen have special requirements for germination (1, 2, 3). Pollen tubes are likewise sensitive to temperature, moisture, sugar concentrations, pH, growth substances, trace elements, etc. Most pollen, if given the correct medium, will produce tubes within a few hours. A standard method for germinating pollen might well be adapted for testing the growth-promoting properties of any number of commercial preparations.

Since gibberellic acid stimulates the germination and growth of *Lilium* and *Petunia* pollen, it may be possible to overcome some of the physiological incompatibilities characteristic of certain clones of these species. Further studies on the effect of gibberellic acid in relation to incompatibilities in flowering plants are contemplated.

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Note

A SIMPLE METHOD FOR THE RECRYSTALLIZATION OF $BaS^{35}O_4$[1]

Val F. Cotty[2] and Leonard H. Weinstein

In the course of a study on sulfur metabolism in house flies it became desirable to separate and measure the amount of $S^{35}O_4^{=}$ produced by flies when they were fed cystine-S^{35} and methionine-S^{35}. An isotopic dilution method was used in which a known amount of nonradioactive $SO_4^{=}$ was added to the unknown sample. Barium chloride was then added in excess and the precipitated barium sulfate removed by centrifugation or filtration. Radioactive contaminants, however, were sometimes co-precipitated with the $BaSO_4$. Such contaminants result in an elevated specific activity and a consequent error in the calculation of the amount of $S^{35}O_4^{=}$ present. The following method of recrystallizing $BaSO_4$ was developed to free the precipitate of radioactive contaminants.

The precipitated barium sulfate-S^{35} (usually 100 mg.) was washed twice with water and transferred to a flask containing 5 ml. of a 20 per cent (w/v) solution of the disodium salt of ethylenediaminetetraacetic acid (EDTA) in 7.4*N* NH_4OH. Water was added to bring the volume to 100 ml. and the pH was verified at >10. After heating at 80° C. for 15 to 30 minutes the clear solution was filtered and its reaction adjusted to pH 6 to 7 with 2*N* HCl. A precipitate of $BaSO_4$ formed and settled out following the addition of 7.5 ml. $NiCl_2 \cdot 6H_2O$ solution (10 per cent w/v).

At a high pH barium from $BaSO_4$ is complexed by EDTA. Lowering the pH releases the barium. It was found, however, that the precipitate of $BaSO_4$ carried EDTA with it. Therefore when the pH was lowered $NiCl_2$ solution was added to form the stable nickel-EDTA complex. The nickel-EDTA complex eliminated adsorption of the chelating agent upon the particles of barium sulfate.

To test the method the following experiment was carried out. S^{35}-labeled $BaSO_4$ was prepared with a specific activity of 409 c.p.m./mg. This (255 mg.) was put into solution as described above and a cockroach hydrolyzate prepared from 100 mg. dry roach material was added. The $BaS^{35}O_4$ was precipitated as described above. Three more resolubilizations and precipitations followed. Aliquots for counting were removed after the

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[1] This research was carried out in part through the aid of the Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-492.

[2] Present address: Bristol-Myers Products Division, Research Laboratories, Hillside, New Jersey.

precipitates were washed with ethanol, then with ether, and dried under a heat lamp. The aliquot (5 to 10 mg.) was dissolved in 10 ml. H_2O containing 0.3 ml. of the EDTA+NH_4OH solution. One-ml. aliquots were dried in planchets and counted with a thin window G-M tube. The results of the experiment are given below; these indicate that the method is efficient for the purification of $BaSO_4$ in isotopic dilution experiments.

Sample	Specific activity c.p.m./mg.
Original $BaSO_4$	409
1st Recrystallization	419
3rd Recrystallization	410

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Note

THE REARING OF ASEPTIC ADULT HOUSE FLIES FOR PHYSIOLOGICAL STUDIES[1]

S. MARK HENRY AND VAL F. COTTY[2]

During investigations of sulfur metabolism in house flies, *Musca domestica* L., it became desirable to determine whether the compounds formed in the fly were products of the fly tissue or of microorganisms. Aseptic adult flies were reared by methods we have found to be superior to those reported elsewhere (3).

Newly laid house fly eggs (roughly 2 to 5 ml.) were placed in 0.5 per cent trypsin, pH 9.0, and gently stirred for two hours at approximately 37° C. The eggs were washed five times in tap water (500 ml. each time). They were then placed in filtered, freshly prepared 1 per cent calcium hypochlorite (Fisher Scientific Company Tested Purity Reagent, C.P.) solution for exactly 2.5 minutes and washed twice in sterile distilled water. Approximately 1 ml. or less of eggs was added to 10 ml. of 4 per cent formaldehyde or tincture of Zephiran Chloride (2) in a 30-ml. Erlenmeyer flask. The flask was stoppered with a rubber stopper and was shaken for 30 minutes on a serological shaker. The eggs were washed five times with 10 ml. of sterile distilled water and about 30 eggs were then placed in each tube of sterile medium. These were incubated at 36° C.

The medium consisted of 12 g. Gaines Krunchon cubes dog food,[3] 0.7 g. Fleischmann's dry active yeast and 25 ml. tap water per 6.5-inch × 1.5-inch tube. The tubes of medium were stoppered with cotton plugs and were incubated for 48 hours at 36° C. before they were autoclaved for 5 minutes at 15 lb. pressure. Sterility tests using thioglycollate fluid medium were run on the eggs, pupae, and adults before experimental feeding and adults at the termination of the experiment. Solutions used in sterility tests were incubated for a minimum of 7 days at 36° C. A high percentage of cultures yielded aseptic adults.

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[1] This research was carried out in part through the aid of the Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-492.

[2] Present address: Bristol-Myers Products Division, Research Laboratories, Hillside, New Jersey.

[3] Manufacturer's guaranteed analysis: protein not less than 25 per cent; fat not less than 6 per cent; fibre not more than 5 per cent; ash not more than 10 per cent; moisture not more than 12 per cent. Ingredients: meat and bone meal; processed corn; wheat and bran flakes; soybean oil meal; fish meal (including liver and glands); corn germ meal; wheat germ meal; dried beet pulp; alfalfa leaf meal; dried skimmed milk; Brewer's dried yeast; iodized salt 0.5 per cent; fish liver oil 0.5 per cent; Riboflavin supplement 0.022 per cent; irradiated yeast 0.006 per cent. Vitamin concentrates have been added to provide 3000 U.S.P. units of vitamin A and 1000 U.S.P. units of vitamin D_2 per pound.

The aseptic flies were used for radioactive tracer experiments. The labeled compound was fed in an amino acid solution comparable in composition to approximately 0.75 per cent casein (1, p. 490). The solution contained 5 per cent glucose and 0.1 per cent NaCl in addition to the amino acid. The food was placed in inverted feeders as shown in Figure 1 and autoclaved in a large test tube for 3 minutes at 15 lb. pressure. The flies were permitted to emerge in the rearing tubes. Under CO_2 or cold anesthesia they were transferred aseptically to sterile cotton-stoppered Fernback flasks. The sterile feeders containing the food solution were then suspended from the lips of the flasks with the attached pieces of wire. The flies were permitted to feed for 24 hours. They were then anesthetized, sampled for sterility testing and processed.

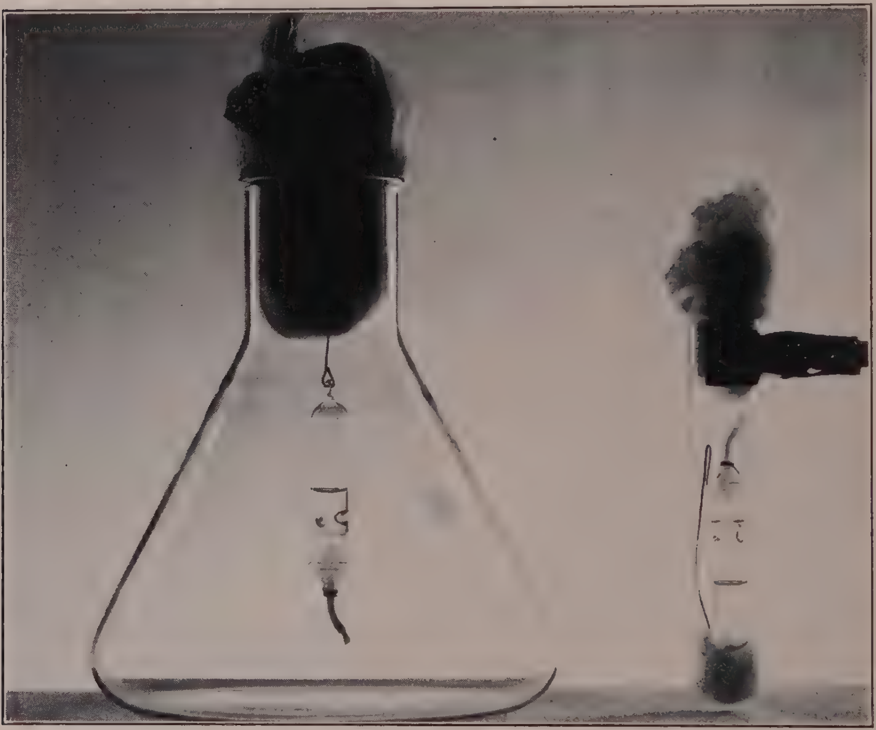

FIGURE 1. Feeder with food prepared for autoclaving (at right) and Fernback flask with feeder for aseptic house fly experiments (at left).

The aseptic adult flies reared by the above method compared favorably in size with the nonaseptic flies routinely reared for insecticide screening at Boyce Thompson Institute.

This note is presented with the view that the procedures described above may prove useful to other workers who may wish to perform physiological or microbiological experiments with aseptic house flies.

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CLEARING FREE AMINO ACID SOLUTIONS OF PLANT EXTRACTS FOR PAPER CHROMATOGRAPHY

Philip H. Plaisted

SUMMARY

The need for removal of interfering substances in ethanolic extracts of plant tissue before determining amino acids by paper chromatography is emphasized and a method of removing these interfering materials is presented. The method for "desalting" the extract utilizes the cation exchange resin Dowex 50-X8, 200-400 mesh, H^+, bedded in 80 per cent ethanol. The ethanolic plant extract is passed through a column of the resin which retains the amino acids. The acidic and neutral amino acids and amides are eluted from the resin with $0.4N$ NH_4OH in ethanol followed by an ethanol and water wash. The basic amino acids are completely removed by percolating $4N$ NH_4OH in water through the resin. Later, the NH_4^+ is removed from the eluting solution by drying under vacuum.

INTRODUCTION

During an investigation of aging in Norway maple leaf tissue, an attempt was made to determine the free amino acids by paper chromatography. When the extract was spotted directly on the papers, it was impossible to identify the amino acids on the chromatograms because of streaking and overlapping. Apparently materials, both ionic and nonionic, extracted with the amino acids were causing this streaking. Various methods have been employed to remove these undesirable materials before spotting the sample on paper. Consden *et al.* (2) used an electrolytic device to remove charged particles from the amino acid or peptide solutions. This was later shown by Stein and Moore (12) to result in a slight loss of amino acids and the conversion of some arginine to ornithine.

Other methods, using ion exchange resins either to "desalt" the amino acid solution before paper chromatography or as a means of chromatographing the amino acids (3, 4, 6, 8, 9, 14, 15), have been developed. The eluting liquids were acidic, either hydrochloric or acetic. These acids, as eluting materials, would be too harsh on naturally occurring amides such as asparagine and glutamine. Hirs, Moore, and Stein (5) developed a chromatographic procedure using Dowex 50 and eluting with formate or acetate. Excellent recovery values were reported for the amino acids in the hydrolyzate of bovine serum albumin, but no mention is made of the amides asparagine and glutamine. Ammonium hydroxide has also been used to elute the amino acids from the resins. Presumably this elutant would not be detrimental to the amides. Bach and Smith (1), Stevenson (13), and Mansford and Raper (7) eluted amino acids from a cation ex-

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change resin with NH_4OH but gave no recovery values. A loss of some amino acids could occur for Racusen and Aronoff (11) reported a loss of arginine, histidine, and lysine when their protein hydrolyzates were eluted from a cation exchange resin with NH_4OH.

Attempts to use some of the techniques mentioned above to "desalt" the Norway maple leaf extract before amino acid chromatography proved futile. A new technique for "desalting" the ethanol extracts and preserving the amides was developed and is reported in this paper.

MATERIALS AND METHODS

The cation exchange resin Dowex 50 was used in the "desalting" technique. This was chosen for it was readily available, would retain the amino acids and amides that were tested, and would quantitatively release them when treated with the appropriate concentrations of the eluting solutions.

The Dowex 50-X8, 200–400 mesh, H^+ was prepared for use by washing with water three to five times. At each wash, the heavy particles were allowed to settle and the fine material was decanted off. The resin was flexed in a 500-ml. burette which had a glass wool plug in the outlet. A 10 per cent NaCl solution (w/v) was passed through it until the effluent liquid was neutral to pH indicator paper. After washing with deionized water until a negative chloride test was obtained, the column was washed with $2N$ HCl. The HCl was passed through until the effluent liquid gave an acid reaction to pH indicator paper. The column was again washed with deionized water. This procedure was continued until the resin had been cycled three times in the Na^+ form and four times in the H^+ form. It was left in the H^+ form and exhaustively washed with water until neutral. After the water wash, the resin was removed from the burette and washed in a large beaker with 80 per cent ethanol (v/v). It was then stored in 80 per cent ethanol until used.

The chromatography column used was a 1×30 cm. glass tube with a fritted glass disc in the bottom. The top of the column had a 12/30 S/T female ground glass joint fused to it. The reservoir attached to the top of the column was a round bottom flask with a 12/30 S/T male joint fused on to the bottom. The column and reservoir are shown in Figure 1 A.

The flow rate of the liquid through the resin was easily regulated in this column by a slight air pressure on the liquid. The amount of pressure was regulated by placing a T-joint in the air line and adjusting the amount of air escaping with a screw clamp. Batteries of three or four columns were operated from one air outlet at the same time.

A column of resin 3 or 5 cm. in height was prepared by washing the resin into the column with 80 per cent ethanol. It was then allowed to settle and a small plug of glass wool was placed on top. The resin was washed before use by passing 15 to 20 ml. of 80 per cent ethanol through it.

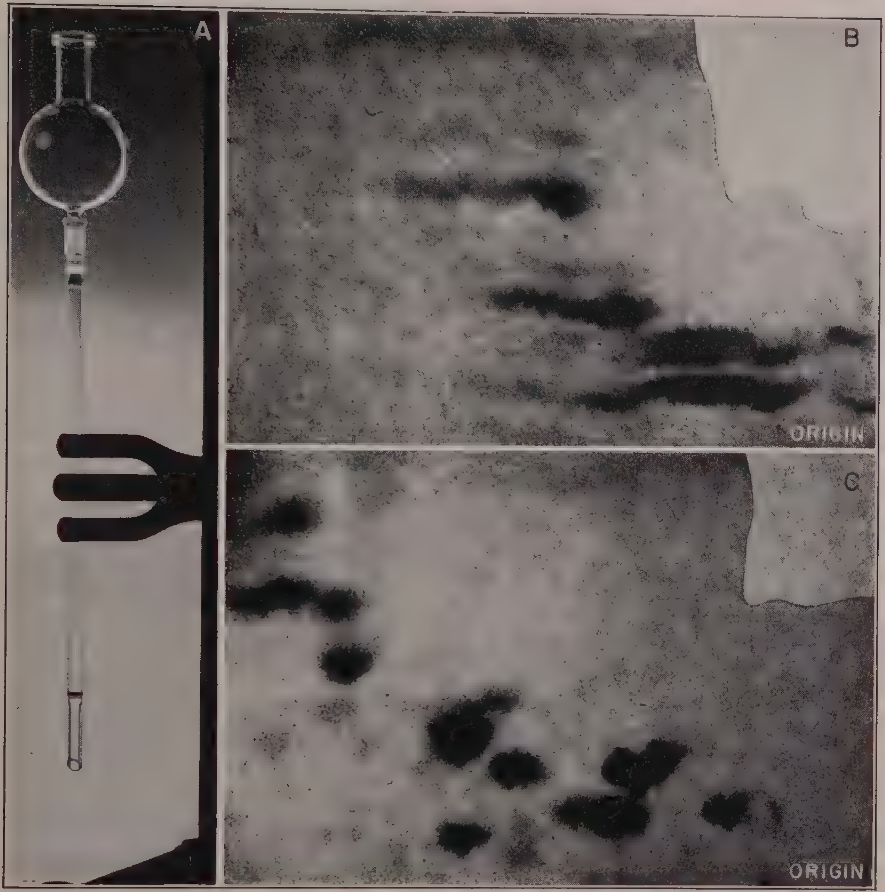

FIGURE 1. (A) Chromatography column with reservoir attached. (B) A free amino acid chromatogram of Norway maple leaf tissue showing the streaking caused by undesirable materials in the leaf extract. (C) Same as B, except the extract was "desalted" before chromatographing.

The amino acids used were obtained from the Nutritional Biochemical Company. These were prepared for testing by dissolving known amounts in 80 per cent ethanol. A typical mixture of amino acids was glutamine, lysine, proline, serine, and the leucines or alanine, γ-aminobutyric acid, arginine, asparagine, aspartic acid, glutamic acid, phenylalanine, and valine. In some cases arginine and glutamic acids were determined separately. The amino acid solutions were prepared in this manner because of ease in separation of the acids on a two-dimensional chromatogram. To carry out an experiment using the standard amino acid solution, an aliquot of the solution was washed into the reservoir of the resin column. A similar portion of the solution equal in volume to that which was passed through the Dowex 50 column was prepared and placed in a flask, then reduced to dryness under vacuum. This evaporation was accomplished with the aid of a Rinco rotating vacuum evaporator at a temperature lower than 40° C. As soon as the acids were dry, they were quantitatively washed from the vacuum flask into a volumetric flask with 10 per cent 2-propanol in water (v/v).

The ethanolic amino acid solution was passed through the resin at a flow rate of 3 ml. per minute. This high flow rate was maintained to minimize the time that the amino acids and resin would be in contact. After the ethanolic amino acid solution had passed through the column, 15 ml. of 80 per cent ethanol was used to rinse the equipment.

The amino acids were eluted from the column with 0.4*N* NH_4OH in 80 per cent ethanol. This ammoniacal ethanol was passed through the resin until pH indicator paper gave a strong basic reaction. When a 0.9×5.0 cm. resin column was used, 40 ml. of the 0.4*N* NH_4OH in ethanol was employed. Less ammoniacal ethanol would be required if the column were decreased in height. The 0.4*N* NH_4OH in ethanol was followed by a 15-ml. wash of 80 per cent ethanol which in turn was followed by 15 ml. of deionized water. The water wash was followed by 15 ml. of 4*N* NH_4OH in water. Finally, the column was washed with 15 ml. of deionized water.

The eluates from the column were combined and taken to dryness under vacuum as previously described. The dried amino acids were quantitatively removed to a 10-ml. volumetric flask with 10 per cent 2-propanol.

The quantity of each amino acid present in the material eluted from the column as well as from the sample that did not pass through the column was determined by paper chromatography. Spots of 30 to 40 μl. were placed on Whatman No. 3 filter paper. The solvents used and the method employed for determining quantitatively the amount of any amino acids on the paper have been described by Porter *et al.* (10). Where comparisons between individual amino acids were made, one-dimensional chromatography employing *n*-butanol-acetic acid-water (90:10:29) (v/v/v) as a solvent was used. Each determination of a given set of amino acids was replicated three to five times. As quantitative paper chromatography is only accurate within 10 per cent (10) recoveries between 90 to 110 per cent were considered quantitative.

Glutamic acid in the presence of 80 per cent ethanol and the cationic ion exchange resin was converted in limited quantities to its γ-monoethyl ester. The isolation and identification of γ-monoethyl glutamate will be described in a later paper. The γ-monoethyl glutamate gave the typical purple color when reacted with ninhydrin. The position of this compound on the chromatogram can be seen in Figure 2. To obtain quantitative recovery of glutamic acid, it was necessary to combine the spot produced by γ-monoethyl glutamate with the one for glutamic acid.

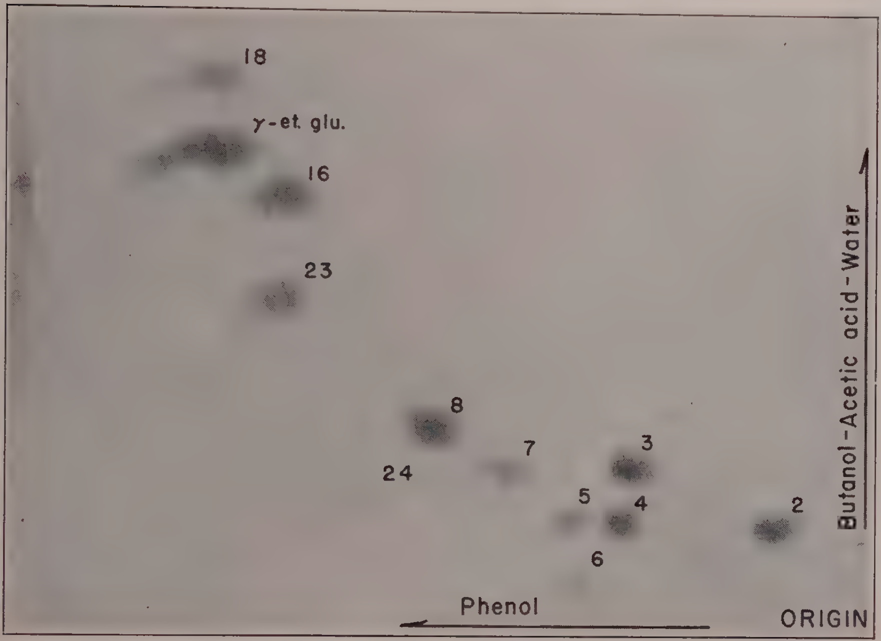

FIGURE 2. Chromatogram showing the location of γ-monoethyl glutamate. The amino acids are: 2. aspartic acid, 3. glutamic acid, 4. serine, 5. glycine, 6. asparagine, 7. threonine, 8. alanine, 16. valine, 18. the leucines, 23. γ-aminobutyric acid, 24. hydroxyproline, γ-et. glu. = γ-monoethyl glutamate.

The effect that a plant extract and the Dowex 50 resin had upon recovery of amino acids was determined by adding known quantities of amino acids to an 80 per cent ethanol extract of Norway maple (*Acer platanoides* L.) leaf tissue. This tissue was chosen for it is impossible to determine quantitatively the free amino acids in it by paper chromatography without first removing the interfering substances. The ethanol extract was divided into two equal portions. A known quantity of nine different amino acids was added to one portion of the extract. Both portions of the ethanol extract were passed through a 0.9 × 5.0 cm. column of Dowex 50. The eluted amino acids were taken to dryness and washed into a volumetric flask as previously described. A volume of 80 per cent ethanol containing the same number and amount of amino acids as added to the leaf ethanol extract was evaporated to dryness and washed into a volumetric flask. The quantity of each amino acid in the leaf extract, the leaf extract with the amino acids added, and the standard amino acid solution was determined by paper chromatography (10).

In order to determine the effect that Dowex 50 had on naturally occurring free amino acids, extracts of potato (*Solanum tuberosum* L.) tuber and

Turkish tobacco (*Nicotiana tabacum* L.) leaf were used. These tissues were chosen since their concentrated extracts gave good amino acid chromatograms. Therefore a direct comparison of the amino acids that were eluted from the Dowex 50 column could be made with those amino acids that had not been on the ion exchange resin. The Turkish tobacco leaf tissue extract required removal of chlorophyll before concentrating and chromatographing. The chromatograms obtained of the free amino acids in the untreated tobacco leaf were fair. Some streaking occurred in those amino acids found near the origin.

Where ethanolic extracts of potato and tobacco were used, the total extract of each was split into two equal portions. One portion was passed through the ion exchange column while the other portion was prepared directly for chromatography. The quantity of each amino acid present in the fraction eluted from the column was compared with the portion of the extract that was not passed through the column. Each treatment was replicated on four different chromatograms. The technique used for paper chromatography has been previously described (10).

EXPERIMENTAL RESULTS AND DISCUSSION

The identification and subsequent quantitative determination of free amino acids in plant tissue by paper chromatography requires an extract free from excessive amounts of sugars and salts. The presence of these interfering compounds causes streaking of the amino acids on the chromatogram as seen in Figure 1 B. The removal of these interfering substances by the technique described in this paper produces the chromatogram seen in Figure 1 C. Both chromatograms were from equal amounts of Norway maple leaf tissue. The need for clearing these interfering substances from the plant extract prior to chromatography is further emphasized by the data shown in Table I. These data were obtained from a chromatogram of Norway maple leaf tissue. Although there was streaking and slight overlapping of spots on the chromatogram of the vacuum dried extract, it was possible to identify those amino acids listed in Table I. However, the extract cleared by the column produced a far superior chromatogram in which there was no streaking or overlapping; therefore it was possible to determine quantitatively the individual spots. Indistinct definition of a spot such as occurred on the chromatogram of the vacuum-dried extract for unknown A produced low optical density values when compared to the "desalted" extract. An overlapping of two spots such as occurred for alanine and threonine on the same chromatogram gave one high reading and one low reading. This demonstrated that it was futile to make quantitative measurements of the amino acids on a chromatogram which had indistinct or overlapping spots.

As the ion exchange resin Dowex 50 used in this "desalting" technique is strongly cationic, it was considered advisable to investigate the effect this resin had upon the amino acids and recovery of amino acids that were in a plant extract.

TABLE I

CONCENTRATION OF FREE AMINO ACIDS IN EXTRACTS OF NORWAY MAPLE LEAF. ONE EXTRACT WAS CLEARED BY ELUTION FROM A DOWEX 50 COLUMN AND THEN CONCENTRATED WHILE THE OTHER WAS CONCENTRATED DIRECTLY BY VACUUM DRYING

Amino acid	Optical density units of the amino acids and standard deviations of their means	
	Desalted by a Dowex 50 column	Vaccum dried
Alanine	0.229±0.002	0.270±0.038
γ-Aminobutyric acid	0.057±0.010	0.073±0.015
Aspartic acid	0.145±0.020	0.088±0.010
Unknown A	0.144±0.009	0.040±0.005
Glutamic acid	0.678±0.008	0.195±0.026
Glutamine	0.040±0.004	0.670±0.068
The leucines	0.370±0.003	0.369±0.049
Phenylalanine	0.030±0.004	0.031±0.006
Proline	0.021±0.003	—
Serine+glycine	0.163±0.014	0.255±0.031
Threonine	0.151±0.006	0.127±0.017
Tyrosine	0.035±0.001	—
Valine	0.259±0.008	0.290±0.006

Separation of amino acids into two groups. The separation of the amino acids into two groups, one containing the basic acids and the other containing the acidic and neutral ones was attempted. This type of scheme was used to facilitate the determination of the basic amino acids and stop them from interfering with other amino acids on a two-dimensional chromatogram. Such a scheme was devised by Mueller *et al.* (8) for protein hydrolyzates. In determining the practicability of such a scheme, 20 amino acids and amides were retained on a Dowex 50 column, then eluted as described and chromatographed one-dimensionally in phenol-water and butanol-acetic acid-water. All the neutral and acidic acids as well as traces of citrulline, lysine, ornithine and tryptophan were eluted with the first liquid, $0.4N$ NH_4OH in 80 per cent ethanol, as seen in Table II. The last eluting liquid, $4N$ NH_4OH in water removed all the arginine, and the bulk of the lysine. It is obvious from Table II that separation of the amino acids into the two groups by this technique cannot be accomplished unless the tissue is known to be free from citrulline, lysine, ornithine and tryptophan.

Recovery of known amounts of amino acids. Recovery values were obtained by making comparisons between amino acids eluted from ion

TABLE II

AMINO ACIDS REMOVED BY CONSECUTIVE ELUTING LIQUIDS FROM A DOWEX 50 COLUMN

Amino acid	Eluting liquid			
	$0.4N$ NH_4OH (ethanolic)	80% Ethanol wash	Water wash	$4N$ NH_4OH (aqueous)
Alanine	+	–	–	–
γ-Aminobutyric acid	+	T	–	–
Arginine	–	–	–	+
Asparagine	+	T	–	–
Aspartic acid	+	+	–	–
Citrulline	+	+	T	+
Glutamic acid	+	T	–	–
Glutamine	+	T	–	–
Glycine	+	+	–	–
Histidine	+	–	–	–
The leucines	+	T	–	–
Lysine	T	T	–	+
Ornithine	+	+	+	+
Phenylalanine	+	–	–	–
Proline	+	–	–	–
Serine	+	T	–	–
Threonine	+	–	–	–
Tryptophan	+	+	+	+
Tyrosine	+	+	–	–
Valine	+	–	–	–

+ = Amino acid present.
– = Amino acid absent.
T = Amino acid present in trace quantity.

exchange resins and standard amino acid solutions. The results shown in Table III indicate that the recovery of all the amino acids tested was quantitative within the limits of the paper chromatographic method used to determine the amount of each acid. These data were obtained by using physiological quantities of amino acids. Most of the amino acids on the chromatograms were present between 5 and 25 μg. Tryptophan had 60 μg. The quantity of asparagine on the paper varied between 35 and 80 μg. Recovery declined from 100 per cent as the amount of acid spotted on the paper decreased. This was caused by the decrease in precision of measurement of the quantity of the paper (10) and not by loss or artifacts produced on the column.

Effect of the resin and eluting materials on the recovery values. Dowex 50, being a strong cationic resin, would presumably have a deleterious effect on the amino acids. To determine if the resin affected the compounds, three amino acids, arginine, γ-aminobutyric acid, and glutamic acid as well as two amides, asparagine and glutamine, were held on the Dowex 50 for various lengths of time. The three amino acids represented the basic, acidic and neutral acids. Asparagine and glutamine are the amides which pre-

TABLE III

RECOVERY OF KNOWN QUANTITIES OF AMINO ACIDS WHICH HAD BEEN PLACED ON A DOWEX 50 COLUMN AND SUBSEQUENTLY ELUTED

Amino acids	Number of times tested	% Recovery and standard deviation of means
Alanine	5	97.6±5.9
γ-Aminobutyric acid	4	94.1±3.3
Arginine	5	104.5±6.3
Asparagine	4	99.1±8.9
Aspartic acid	6	97.4±3.3
Glutamic acid	2	103.9±5.5
Glutamine	4	96.1±4.6
Glycine	6	98.3±4.0
Histidine	1	102.4
The leucines	1	103.2
Lysine	3	100.2±3.8
Phenylalanine	1	107.3
Proline	2	101.3±6.3
Serine	5	95.9±7.2
Threonine	4	97.7±3.0
Tryptophan	1	100.3
Valine	5	95.2±2.5

dominate in plant tissue. The results in Table IV show that the resin causes no deleterious effects to the amino acids tested for at least five hours. A five-hour time interval is more than adequate to pass an alcoholic plant extract through the column. If the volume of the alcoholic plant extract is too large to pass through the resin in less than five hours, two or more columns should be used.

In many instances where time is a limiting factor it is convenient to remove the amino acids from the column and leave them in the eluting liquid. Arginine, γ-aminobutyric acid, glutamic acid, and glutamine were left in the eluting liquid for as long as 72 hours. The recovery in all cases

TABLE IV

RECOVERY OF FIVE AMINO ACIDS FROM DOWEX 50 H^+ COLUMN AFTER DIFFERENT PERIODS OF INCUBATION

Amino acids in contact With resin (hours)	Per cent recovery of amino acids				
	Arginine	Asparagine	γ-Amino-butyric acid	Glutamic acid	Glutamine
2	90	91	96	95	110
4	—	101	100	97	—
5.5	92	—	—	—	100
24	96	91	83	52	65
46.5	81	93	71	51	24

TABLE V

RECOVERY OF AMINO ACIDS ADDED TO AN ETHANOL EXTRACT OF MAPLE LEAF TISSUE BEFORE CLEARING BY SORPTION ON A DOWEX 50 COLUMN. PER CENT RECOVERY IS BASED UPON OPTICAL DENSITY OF THE NINHYDRIN REACTION PRODUCTS OF THE STANDARD AMINO ACIDS

Treatment	Optical density values of amino acids and standard deviation of means								
	Alanine	γ-Amino-butyric acid	Aspartic acid	Glutamic acid	Glutamine	Glycine	Serine	Threonine	Valine
Maple extract with amino acids added	0.916 ±0.010	0.325 ±0.015	0.208 ±0.017	1.187 ±0.036	0.408 ±0.034	0.408 ±0.009	0.746 ±0.074	0.414 ±0.041	0.349 ±0.023
Maple extract alone	0.451 ±0.031	0.141 ±0.015	0.046 ±0.008	0.822 ±0.071	0.086 ±0.008	0.045 ±0.004	0.411 ±0.008	0.152 ±0.007	0.077 ±0.003
Maple extract with amino acids added minus the maple extract alone	0.465	0.184	0.162	0.365	0.322	0.363	0.335	0.262	0.272
Amino acids alone	0.426 ±0.009	0.196 ±0.011	0.176 ±0.008	0.372 ±0.029	0.382 ±0.028	0.404 ±0.019	0.347 ±0.017	0.289 ±0.021	0.290 ±0.013
% Recovery	109	94	92	98	84	90	97	91	94

was quantitative; therefore, we can assume that there is no adverse effect by the eluting liquid on the amino acids.

Recovery of standard amino acids added to a plant extract. The recovery values obtained when standard amino acids are added to a plant extract and the extract is then cleared by the ion exchange technique are shown in Table V. The recovery value for glutamine is low, 84 per cent. This is believed caused by the small amount of glutamine which occurs in this age tissue. The measurement coefficient of variation for glutamine at the level reported in Table V is about 20 per cent (10). Higher recovery values were obtained for aspartic acid, glycine, and valine than glutamine although all of these were present on the chromatogram in small amounts; therefore the coefficient of variation for these was high. Considering these facts, it is doubtful that the combination of resin, ethanol and plant extract had any effect on recovery of the standard amino acids.

Recovery of naturally occurring amino acids from a plant extract. The 80 per cent ethanol extract of potato tubers and Turkish tobacco leaf tissue were used to measure the recovery of amino acids in naturally occurring tissue. These tissues were chosen because they were believed to give good amino acid chromatograms without "desalting" the ethanol extracts. Therefore, there would be a direct comparison between those amino acids that were recovered from the column and those in the vacuum-dried extracts. Although recovery of 15 amino acids was determined in the potato extract and 12 in the tobacco leaf extract, this did not include all the amino acids that were present on the chromatograms. Those amino acids present in small quantitites as well as spots that could not readily be identified were omitted. In the potato extract a spot that corresponded chromatographically to methionine sulfoxide was determined but no attempt was made to establish the true identity of this compound.

The recovery values for the potato tuber amino acids are shown in Table VI. All recovery values were considered quantitative.

The recovery values of the Turkish tobacco leaf amino acids in the vacuum-dried extract and those eluted from the column are shown in Table VII. Those amino acids near the origin on the chromatogram of the vacuum-dried sample were streaked and mingled with many unknown pink, orange, and yellow spots. The chromatogram of the sample, after it had been "desalted," showed no streaking nor unknown colored spots. This is believed to be the reason why such apparent recovery values as 461 per cent were obtained for asparagine and 180 for serine.

The amino acid recovery values shown in Tables VI and VII bring out two points. The recovery found was never less than that obtained with uncleared extracts. Some amino acids gave recovery values far above that obtained with uncleared extracts. Therefore, it can be concluded that removal of undesirable materials from the extract will be beneficial in quantitative determination of amino acids by paper chromatography.

TABLE VI

Comparison of the Free Amino Acid Composition of Two Samples of Potato Extracts. One Sample Was Cleared of Undesirable Materials by Sorption of the Amino Acids on a Dowex 50 Column and Subsequently Eluting with NH_4OH in Ethanol, the Second Was Vacuum Dried

Amino acids	Optical density values of amino acids and standard deviation of means		% Recovery of vacuum-dried sample
	Extract eluted from resin	Vacuum-dried extract	
Alanine	0.134 ± 0.004	0.135 ± 0.008	99
γ-Aminobutyric acid	0.127 ± 0.010	0.124 ± 0.025	102
Arginine	0.118 ± 0.024	0.123 ± 0.019	96
Asparagine	0.665 ± 0.041	0.679 ± 0.108	98
Aspartic acid	0.040 ± 0.001	0.040 ± 0.021	100
Glutamic acid	0.323 ± 0.021	0.321 ± 0.042	101
Glutamine	1.198 ± 0.058	1.198 ± 0.007	100
Glycine	0.034 ± 0.002	0.031 ± 0.004	110
The leucines	0.160 ± 0.012	0.160 ± 0.006	100
Lysine	0.034 ± 0.009	0.038 ± 0.001	90
Methionine sulfoxide	0.032 ± 0.001	0.029 ± 0.005	110
Serine	0.119 ± 0.009	0.119 ± 0.008	100
Threonine	0.134 ± 0.008	0.134 ± 0.007	100
Tyrosine	0.043 ± 0.017	0.044 ± 0.007	98
Valine	0.428 ± 0.032	0.430 ± 0.016	100

TABLE VII

Comparison of the Free Amino Acid Composition of Two Samples of Tobacco Extract. One Sample Was Cleared of Undesirable Materials by Sorption of the Amino Acids on a Dowex 50 Column and Subsequently Eluting with NH_4OH in Ethanol, the Second Was Vacuum Dried

Amino acids	Optical density values of amino acids and standard deviation of means		% Recovery of vacuum-dried sample
	Extract eluted from resin	Vacuum-dried extract	
Alanine	0.268 ± 0.003	0.241 ± 0.014	111
γ-Aminobutyric acid	0.231 ± 0.035	0.228 ± 0.018	101
Arginine	0.025 ± 0.003	0.026 ± 0.002	96
Asparagine	0.586 ± 0.091	0.127 ± 0.019	461
Aspartic acid	0.103 ± 0.015	0.109 ± 0.019	94
Glutamic acid	0.482 ± 0.037	0.496 ± 0.013	97
Glutamine	0.264 ± 0.009	0.242 ± 0.013	109
Glycine	0.023 ± 0.003	0.016 ± 0.001	144
Lysine	0.031	0.030	103
Serine	0.214 ± 0.026	0.119 ± 0.018	180
Threonine	0.079 ± 0.002	0.065 ± 0.004	122
Valine	0.028 ± 0.003	0.031 ± 0.003	90

This method for purifying free amino acid extracts for paper chromatography was not detrimental to the amino acids tested when used under the conditions recommended. It has the advantage of ease, speed, and preservation of the amides. The technique is briefly outlined below.

DESALTING

1. The 80 per cent ethanol extract of plant tissue is passed through a 1×5 cm. column of Dowex 50 H^+ at a flow rate of 3 ml. per minute.
2. The column is rinsed with 80 per cent ethanol.
3. The amino acids are eluted from the column by passing the following solutions through the resin:
    (a) 40 ml. of $0.4N$ NH_4OH in 80 per cent ethanol,
    (b) 15 ml. of 80 per cent ethanol,
    (c) 15 ml. of deionized water,
    (d) 15 ml. of $4N$ NH_4OH in water, and
    (e) 15 ml. of deionized water.
4. All eluates are collected in one container and taken to dryness.
5. The dried amino acids are quantitatively taken up in a small volume of 10 per cent 2-propanol in water.

CHROMATOGRAPHY

Quantitative determination of glutamic acid requires combining the spot produced by glutamic acid and its ester, γ-monoethyl glutamate.

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SOME BIOCHEMICAL CHANGES DURING DEVELOPMENT AND AGING OF ACER PLATANOIDES L. LEAVES

Philip H. Plaisted

SUMMARY

The changes in protein, alcohol-soluble nitrogen and free amino acids were determined during the ontogeny of a leaf from *Acer platanoides* L. The amount of protein per leaf increases during the late spring and early summer, dropping to a constant level during the summer, and rapidly decreasing as the leaf reaches old age. The amount of soluble nitrogen in a leaf follows a pattern similar to the protein content except for the last few days before abscission, at which time it increases.

The 13 amino acids analyzed fell into three different groups. The three groups were based upon the change in amount per leaf of certain amino acids as old age approaches. The content per leaf of γ-aminobutyric acid and serine plus glycine followed the protein pattern described above. Aspartic and glutamic acids as well as alanine decreased in concentration as the season progressed. The remaining amino acids that were measured, glutamine, phenylalanine, threonine, the leucines and valine, increased as the leaf aged. The possible significance of the varying amounts of amino acids in aging maple leaves is discussed.

INTRODUCTION

The problem of aging in plant tissue is imperfectly understood. Crocker (6) described some of the changes that take place in plants as they age. The general biochemical trend is from anabolism to catabolism. Walkley (23) demonstrated that barley leaves could synthesize protein at all ages but the rate of synthesis decreased with age. If the protein synthetic rate decreases, the protein concentration will decrease as the leaf ages.

Many materials present in plant tissue are soluble in 80 per cent ethyl alcohol. This fraction contains different nitrogenous constituents among which are the unbound amino acids. The concentration of soluble nitrogen in the alcohol fraction is directly related to the protein concentration; the higher the concentration of soluble nitrogen the higher the protein concentration up to a maximum (16).

Many biochemical changes that are believed to occur in aging tissue have been studied in cut leaves. Some catabolic reactions have been characterized by analyzing the cut leaves at different time intervals after harvest. The change in the composition of the alcohol-soluble fraction of cut leaves has been studied by Yemm (28, 29). He found amino acids and amides increased in concentration as the time between cutting and analysis lengthened. Presumably these alcohol-soluble materials arose from two sources, some synthetic activity (5, 30) in conjunction with marked pro-

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tein catabolism. Other workers (7, 26, 27), using a similar technique, reported similar results.

The present work was undertaken to study some of the gross biochemical changes as well as the changes in the free amino acids that accompany aging in the maple leaf tissue. Wood and Cruickshank (26) presented evidence that cut Kikuyu grass preferentially oxidized some of the free amino acids as respiratory substrates after depletion of available carbohydrates. Evidence for this same type of oxidation was investigated in the maple leaf.

EXPERIMENTAL METHODS

Sun and shade leaves were collected periodically during the spring, summer and fall from a large tree of *Acer platanoides* L. (Norway maple). Three replicates were harvested at each sampling. All samples were taken from three limbs projecting from the east side of the tree. The last sample was collected during the time of natural abscission, 200 days after leaf growth started. Each sample contained similar size leaves and weighed between 25 and 40 grams. The leaves were quickly weighed, counted, washed, and frozen. The moisture was removed from the leaves by lyophilizing. The dried leaves were weighed and stored in a container over anhydrous $CaCl_2$.

The tissue was prepared for chemical analysis by grinding in a Wiley mill to pass a 40-mesh screen. Total nitrogen and the alcohol-insoluble nitrogen (recorded as protein when multiplied by 6.25) were determined by the conventional Kjeldahl technique (25). Alcohol-soluble nitrogen per leaf was the difference between the total and alcohol-insoluble nitrogen.

The solution used for free amino acid determinations was obtained by extracting 2.5 grams of the ground dried tissue five times with warm (50° C.) 80 per cent ethanol. To prepare the extract for chromatography, it was passed through a 1×5 cm. column of Dowex 50-X8 200–400 mesh H^+ bedded in 80 per cent ethanol. The flow rate was adjusted to 1 to 1.5 ml. per minute. The amino acids were eluted from the resin with HCl as described by Mueller *et al.* (11). This technique quantitatively converted glutamine to glutamic acid.

Later, a method of eluting the amino acids from the ion exchange resin with ammonium hydroxide was developed (17). This new method preserves the amides and allows them to be determined quantitatively by paper chromatography. The amides and acidic amino acids of the leaf tissue were determined on a sample of the alcohol extract cleared by the ion exchange-ammonium hydroxide technique (17). The eluate from the ion exchange resins was reduced to dryness in vacuum and made up to a volume of one ml. in a calibrated tube. The amino acids on a chromatogram of the leaf tissue extract were located by comparing them to chromatograms of known amino acids. Also compounds other than ninhydrin were used to show the presence of some amino acids (4, pp. 75–126). Chromatograms of hydrolyzed samples of the extract, as well as co-chromatograms of certain of the amino acids were employed as identification tools. The quantity of amino acid per sample was determined by the paper chromatography technique described by Porter *et al.* (18).

The amino acid composition of the leaf protein was established by hydrolyzing a sample of the dried tissue. Approximately 140 mg. of the dried, alchol-extracted residue was refluxed with 15 ml. of 6*N* HCl for 21 hours. After cooling, the sample was cleared (11), reduced to dryness five times in vacuum, then made to a volume of one ml. The amino acids present in the hydrolyzate were determined by the method described in the preceding paragraph.

The isolated chloroplasts were prepared from fresh maple leaves by a modification of the technique developed by McClenden (9). The deveined blades from 49 grams of maple leaves were slurried in half-molar sucrose buffered at pH 6.5 by grinding 30 seconds in a VirTis "45" Homogenizer. The resulting green slurry was filtered through a thick layer of glass wool. The green filtrate contained whole chloroplasts, broken chloroplasts, and cell debris. The cell debris was removed by centrifuging for 30 minutes at five times gravity. The chloroplasts were thrown down by recentrifuging the supernatant at 887 times gravity. Microscopic examination of the green pellet showed whole and broken chloroplasts with only traces of cell debris. The chloroplasts were washed with water and then dried in vacuum over anhydrous $CaCl_2$. The chloroplasts were hydrolyzed and the amino acids determined as previously described.

The ammonia present in the leaf tissue was determined by the technique described by Vickery and Meiss (22). As only small amounts of ammonia were present, it was distilled into HCl and determined with Nessler's reagent as described by Thompson and Morrison (21).

Sugars present in the tissue were extracted with 80 per cent ethanol. The extract was evaporated to dryness in vacuum and the resulting residue taken up with warm water. The water solution was cleared by passing through a 1×5 cm. column of Dowex 50-X8 200–400 mesh H^+ resin followed by a 1×15 cm. column of IR 45-X3.5 16–50 mesh OH^+ resin. The total carbohydrates were determined as sucrose by the anthrone method described by Yemm and Willis (31). Chromatography of duplicate extracts showed both glucose and sucrose present with the latter in higher concentration. Starch was determined on the alcohol-insoluble residue as reported by McCready *et al.* (10).

EXPERIMENTAL RESULTS AND DISCUSSION

The greatest amount of protein in a leaf occurred early, about 68 days after growth started as shown in Figure 1 A. As the season progressed, the

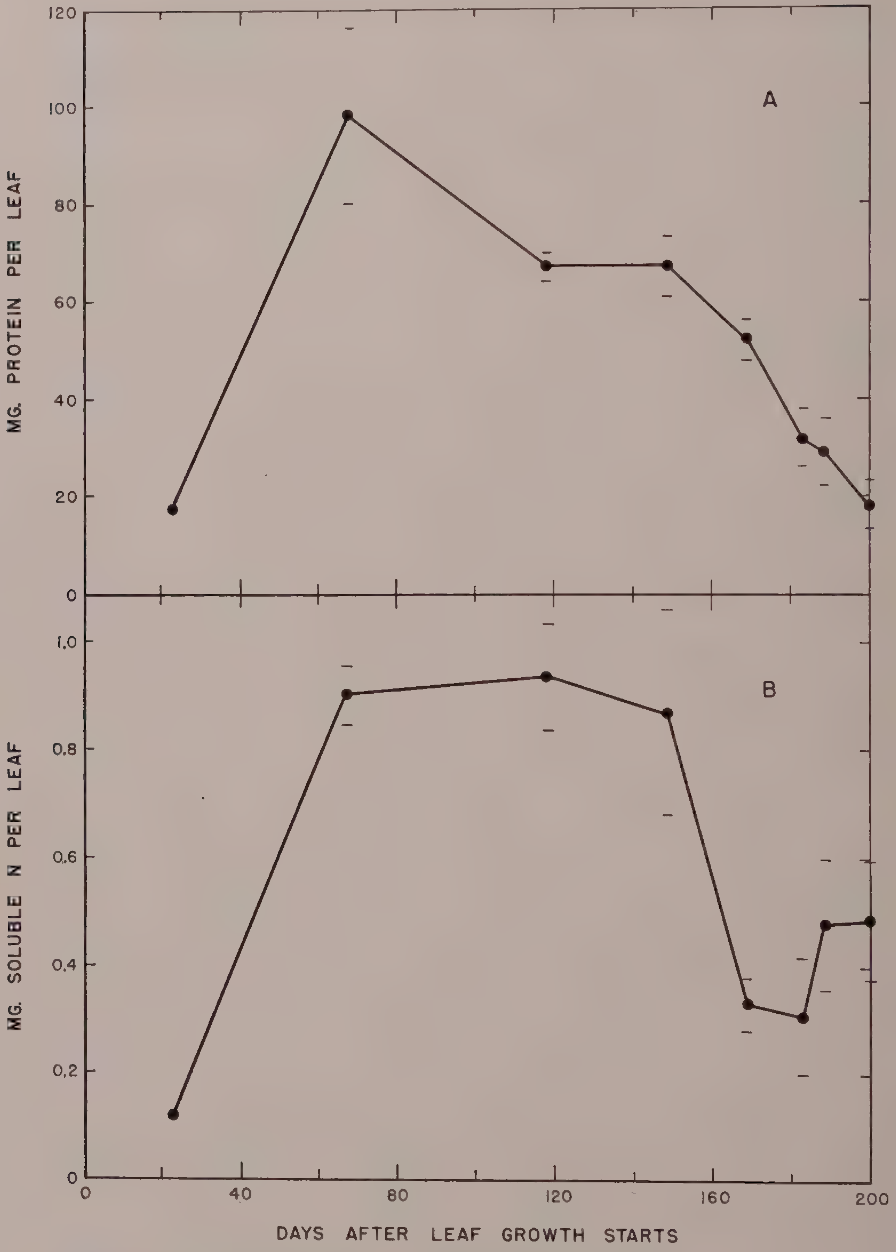

FIGURE 1. The change in nitrogen content during the ontogeny of a leaf of *Acer platanoides* L. (A) Protein (alcohol-insoluble N × 6.25). (B) Alcohol-soluble nitrogen. The short lines above and below each datum point indicate the standard error of the replicate means.

protein content per leaf declined. Approximately 50 days before natural abscission, there was a marked drop in protein. This is in agreement with the findings of Sampson and Samisch (19) in oak and Olsen (15) in beech.

During the summer, the amount of alcohol-soluble nitrogen per leaf, when plotted as a function of time followed a curve similar to that seen in Figure 1 A for protein. Figure 1 B shows that the decline in soluble nitrogen content stops about 30 days before abscission, then shows a slight increase.

The initial increase in both protein and alcohol-soluble nitrogen is probably related to new cell formation and cell enlargement. It is well known that meristematic cells are rich in protein. Many workers (1, 2, 3), have demonstrated that enlarging cells synthesize protein. Presumably all of the maple leaf cells do not mature simultaneously. The different cells of the leaf such as those found in meristematic margins may be undergoing cell division and enlargement after other leaf cells have reached maximum size. Such a situation exists in the squash fruit (20). The cells which are undergoing division and enlargement would contribute more protein nitrogen to the leaf analysis than others. Leaf growth probably does not continue for the 68 days shown in Figure 1 A which is the time of maximum protein content per leaf. More frequent collection of samples during the first of the growing season would be required to establish this supposition.

The decrease in the concentration of leaf protein after the maximum had been attained has an analogy in root growth. The youngest section of the root is nearest the tip. As the newly formed cells enlarge, the protein concentration increases; as the cells reach maximum size the protein content decreases (2). The concentration of protein per leaf follows a similar trend.

The greatest amount of alcohol-soluble nitrogen occurred either in conjunction with high protein concentration, or just prior to abscission. Figure 1 B shows the relationship between soluble nitrogen concentration and age of the leaf. Presumably the first high peak of soluble nitrogen was associated with protein synthesis. This is in agreement with the results of Phillis and Mason (16) who found that the concentration of protein increased as the crystalloid (alcohol-soluble) nitrogen increased up to a maximum. The increase in soluble nitrogen that took place just before abscission may have been associated with proteolysis of the chloroplast proteins. Although no visible loss of chlorophyll occurred 30 days prior to abscission, accelerated breakdown of the chloroplast proteins may have started at this time. These alcohol-soluble products of proteolysis would either be respired or translocated out of the leaf under normal conditions. The fact that the alcohol-soluble products showed a slight increase indicated that an abnormal situation had arisen in the leaf. A block in the translocation system would cause a build-up of soluble products. Lee (8) noticed accumulation of tyloses in the vessels of *Acer pseudoplatanus* just before abscission formation. Although the conventional theory associates upward translocation with the vessels, a partial block of some type may exist in the phloem which would decrease translocation out of the leaf.

The relative amount of the thirteen free amino acids found in the soluble nitrogen fraction of a maple leaf was estimated. Table I shows how the amount of each amino acid varied as the leaf aged. As serine and glycine were not separated chromatographically in all cases they were measured together and reported as serine plus glycine. Separate determinations on a few samples showed serine was about 10 times more abundant than glycine. Several other free amino acids were present in addition to those shown in Table I. Lysine and arginine were found on all the chromatograms but only in trace quantities. The arginine content per leaf may have been higher than reported here. Oland and Yemm (14) found that a final hot water extraction was necessary for the removal of arginine from apple stem and root tissue. No hot water extraction was undertaken with the maple leaf tissue. Asparagine was more readily visible on chromatograms taken from samples harvested within the last 25 days of the season than on those from earlier samples. Even the highest concentration obtained for asparagine was too low to measure. As the season progressed, two unidentified ninhydrin-reacting spots arbitrarily labeled A and B were noticed. Both compounds, A and B, could be retained on a cation exchange column and were resistant to dilute HCl and NH_4OH. Compound A had

TABLE I

CHANGES IN THE AMINO ACID CONTENT PER LEAF OF ACER PLANTANOIDES DURING THE GROWING SEASON. THE PLUS OR MINUS VALUES ARE THE STANDARD ERROR BETWEEN REPLICATE MEANS

Amino acids	Amount of predominant amino acids and NH_3 in μg./leaf							
	Days after leaf growth starts							
	23	68	118	149	169	183	189	200
Alanine	107	66	86±16	62±1	26±1	18±4	37±1	27±9
Aspartic acid	17	18	33	10	8	4	5	T
Glutamic acid	224	104	67±6	47	61	—	61	23
Glutamine	58	15	39±9	25	53	92	125	126
γ-Aminobutyric acid	80	32	22±4	144±4	76±2	36±1	49±2	37±2
The leucines	15	—	12	10	5	20	45±1	57
Phenylalanine	12	—	T	T	T	T	11	18
Serine plus glycine	10	45	43±5	91±5	71±1	34±1	32±5	45±2
Threonine	19	7	14±1	17±2	12±3	10±1	20±3	28±1
Tyrosine	8	—	T	T	T	T	T	T
Valine	T	5	9±1	6	5	13±3	23±3	41±15
NH_3	—	—	—	—	—	11	18	27

T = Trace quantity not measured.
— = No determination made.

the same Rf in phenol as threonine and a slightly higher Rf in butanol-acetic acid-water than alanine. The second unknown, B, had the same Rf in phenol as glutamic acid and a slightly higher Rf in butanol-acetic acid-water than unknown A. Visual estimations of the concentration of both compounds indicated they were most prevalent during the summer months. Many other compounds were visible in trace quantities but no attempt was made to measure or identify them.

The free amino acids that were found in the maple leaf can be divided into three different groups. The three groups were based upon the change in amount per leaf of various amino acids as old age approaches, Table I. The first group, composed of γ-aminobutyric acid (γ-AB) and serine plus glycine followed a trend reminiscent of that seen for protein, Figure 1 A, when concentration per leaf was plotted as a function of time. Aspartic and glutamic acids, as well as alanine decreased in amount as the leaf aged. The remaining amino acids, glutamine, the leucines, phenylalanine, threonine, and valine showed a build-up as abscission approached.

The content per leaf of γ-AB and serine plus glycine parallels that for protein during the summer. As the protein content decreased, the amount per leaf of these amino acids also decreased. The amount of γ-AB present in the leaf at any time may be related to the concentration of glutamic acid and glutamine present since presumably there is a chemical relationship between the three. The decarboxylation of glutamic acid would yield γ-AB. Naylor and Tolbert (12) have shown that young barley plants will convert glutamic acid to γ-AB under anaerobic conditions, but under aerobic conditions the barley plants converted glutamic acid to glutamine, small amounts of γ-AB, and malic acid. An enzymatic system that converts glutamic acid to γ-AB may also be present in maple. As the growing season progresses the competition for available glutamic acid between protein synthesis, glutamine formation and conversion to other amino acids may reduce that utilized for γ-AB synthesis and/or the activity of the enzyme which catalyzes the glutamic$\rightleftharpoons\gamma$-AB reaction may decline. In either case, the concentration of γ-AB per leaf would decline as the leaf aged.

The amount of aspartic and glutamic acids as well as alanine decreases as the leaf ages. These three amino acids occupy a unique position in the metabolism of the cell. Although the details have not been worked out, the evidence indicates that the acidic amino acids and alanine may act as precursors of other amino acids (24) as well as being the first ones formed in photosynthesis (13, 24). Aging is apparently associated with a decreased synthetic ability and loss of chlorophyll in extreme age. Both of these factors would decrease the concentration of three "key" amino acids in a cell. Although proteolysis would liberate a considerable amount of these three "key" amino acids, Table II, the leaf does not have an adequate

TABLE II

RELATIVE AMOUNTS OF PREDOMINANT AMINO ACIDS IN LEAF TISSUE AND CHLOROPLAST HYDROLYZATES FROM ACER PLATANOIDES

Amino acids	Amount expressed as per cent of total present in one gram dry material		
	Tissue analyzed		Chloroplast extracted from leaves 78 days old
	Age of leaf tissue hydrolyzed		
	149 days*	200 days**	
Alanine	6.8	7.4	7.8
Arginine	7.1	4.6	5.8
Aspartic acid	5.0	6.2	1.4
Glutamic acid	11.4	14.5	12.8
The leucines	20.8	20.1	21.9
Lysine	8.0	9.2	8.2
Phenylalanine	11.2	3.7	6.3
Proline	—	—	1.5
Serine plus glycine	12.7	18.8	16.7
Threonine	5.3	6.2	6.1
Tyrosine	3.1	2.2	3.3
Valine	8.7	7.9	7.4

* Leaves were green at end of 149 days of growth.
** Leaves were yellow at end of 200 days of growth.

supply to meet all needs. These facts may explain why the three "key" amino acids decrease in concentration as the leaf ages.

The amino acid composition of the leaf protein, including chloroplasts, from mature and senescent leaves as well as the amino acid composition of isolated chloroplasts is shown in Table II. The different tissues contain essentially similar amounts of the different amino acids. At any given time during the last 40 days of the leaf's life, proteolysis would liberate a higher proportion of glutamic acid, the leucines, phenylalanine, and serine plus glycine than the others.

The content per leaf or glutamic acids would be lowered by the many metabolic needs for this amino acid. Naylor and Tolbert (12) demonstrated that radioactive glutamic acid was aerobically converted to malic acid in cut young barley leaves. Glutamic acid as well as other amino acids such as aspartic acid and alanine and those which are chemically related could be converted to acids of the Krebs cycle and therefore would be depleted from the leaf. The build-up of glutamine and ammonia in the aging leaf tissue of maple, Table I, was similar to the increase in glutamine and ammonia found in starving barley leaves by Yemm (28, 29) and starving Kikuyu grass by Wood *et al.* (27). The increase in ammonia concentration may indicate that some of the amino acids were used as substrates for respiration even though carbohydrates were still present in the leaf (Table III). Wood and Cruickshank (26) presented evidence that amino

TABLE III

CARBOHYDRATE CONTENT OF ACER PLATANOIDES LEAVES AT DIFFERENT STAGES OF DEVELOPMENT

Days after leaf growth starts	Amount in mg./leaf	
	Sucrose	Starch
149	21.0	11.9
169	16.8	8.6
189	12.1	5.9
200	7.6	6.0

acids may be preferentially oxidized under aerobic conditions in starving Kikuyu grass leaves. A similar reacion could take place in sensecent maple leaves. Some of the amino acids resulting from proteolysis and limited synthesis could be preferentially oxidized through the Krebs cycle. The remaining amino acids would be the less easily oxidizable ones such as the leucines, phenylalanine, threonine, and valine. These, although translocation is believed slowed, could be relocated to the main body of the plant.

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PLANT GROWTH REGULATING ACTIVITY OF 3-INDOLEMETHANESULFONIC ACID

A. J. Vlitos[1] and Thomas S. Hitchcock[2]

SUMMARY

3-Indolemethanesulfonic acid (IMSA) was found to be an active plant growth regulator in a wide variety of growth regulant assays. The compound induced leaf modification, stem curvature, and proliferation of intact tomato and bean seedlings, promoted growth of *Avena* first internode sections, induced curvature of slit pea sections, and suppressed the elongation of roots of cucumber seedlings. In preliminary weed control experiments, IMSA at 5 and 10 lb. per acre applied as a pre-emergence herbicide controlled both grass and broad-leaved weeds in broccoli, mustard, radish, and cabbage plantings with no injury to radish and slight injury to the remaining crops. Applied as a post-emergence herbicide, IMSA at 10 and 20 lb. per acre controlled broad-leaved and grass weeds in soybean, corn, and oat crops with no injury to oats, slight injury to corn, and severe damage to soybeans.

INTRODUCTION

Although it was suggested as early as 1938 (3) that an indole derivative to be effective as a plant growth regulator should possess a terminal carboxyl group in the side chain, or a group readily convertible to a carboxyl group, it has become increasingly evident in recent years that some derivatives of indole which are effective as plant growth regulators do not meet this requirement.

3-Indolemethanesulfonic acid (IMSA), which differs from 3-indoleacetic acid (IAA) in that a sulfonic acid group replaces the carboxyl, has been reported to be active in the pea curvature, *Avena* straight-growth, and flax root bioassays (7). Erdtman and Pettersson (1) found IMSA to be inactive in the *Avena* coleoptile curvature test while Wieland *et al.* (8) reported that IMSA was an auxin antagonist. The present study describes the plant growth regulating activity of IMSA[3] in a wide range of bioassays and in selective weed control experiments.

RESPONSE OF TOMATO SEEDLINGS TO IMSA

Six-week-old tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) seedlings grown in composted soil were sprayed with $10^{-2}M$ to $10^{-5}M$ aqueous solutions of IMSA or IAA. Control seedlings were sprayed with equivalent amounts of water. Treated seedlings were held in the greenhouse (22° C. to 25° C.). Stem curvature responses were recorded after 1, 4 and 8 days. Modification of leaf structure was rated after 8 and 14 days, while proliferation of stems was recorded 14 days following initial treatment. A modified system of Hitchcock and Zimmerman (2) was employed to evaluate the various physiological responses. Comparative activities of IMSA and IAA in the tomato bioassay are recorded in Table I. Results are presented as average responses for three replicate treatments per experiment in three separate experiments performed at different times.

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[1] Senior Fellow in Plant Physiology, Union Carbide Chemicals Co. Fellowship.

[2] Research Assistant in Plant Physiology, Union Carbide Chemicals Co. Fellowship.

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IMSA at concentrations of 10^{-2} and $10^{-3}M$ induced stem curvature and leaf modification. At the highest dosage the compound caused slight proliferation of stem tissues. IAA at 10^{-2} and $10^{-3}M$ induced stem curvatures and slight proliferation but did not cause modification of leaf form. Recovery from curvatures was much more rapid in plants treated with IAA than it was in plants treated with IMSA. Fourteen days after treatment, plants sprayed with IAA appeared normal except for slight proliferation of stems, whereas plants treated with IMSA ($10^{-2}M$) had produced leaves with modified morphological patterns and stems with elongated internodes (Fig. 1).

TABLE I

COMPARATIVE ACTIVITY OF IMSA AND IAA FOR INDUCING RESPONSES IN INTACT TOMATO SEEDLINGS

Treatment applied		Response of seedlings*					
Material	Molarity	Stem curvature after			Leaf modification after		Proliferation after 14 days
		1 day	4 days	8 days	8 days	14 days	
H_2O	—	0	0	0	0	0	0
IMSA	10^{-2}	++	++	++	++	++	+
	10^{-3}	+	+	0	0	+	0
	10^{-4}	0	0	0	0	0	0
	10^{-5}	0	0	0	0	0	0
IAA	10^{-2}	++	+	0	0	0	+
	10^{-3}	++	0	0	0	0	+
	10^{-4}	0	0	0	0	0	0
	10^{-5}	0	0	0	0	0	0

* 0 = No response.
+ = 5° to 10° stem curvature; slight leaf modification; slight proliferation.
++ = 11° to 20° stem curvature; moderate leaf modification; moderate proliferation.
+++ = >20° stem curvature; severe leaf modification; severe proliferation.

In subsequent experiments IMSA at 500 and 750 p.p.m. caused extension of internodes and a consequent increase in over-all height of tomato seedlings (Table II).

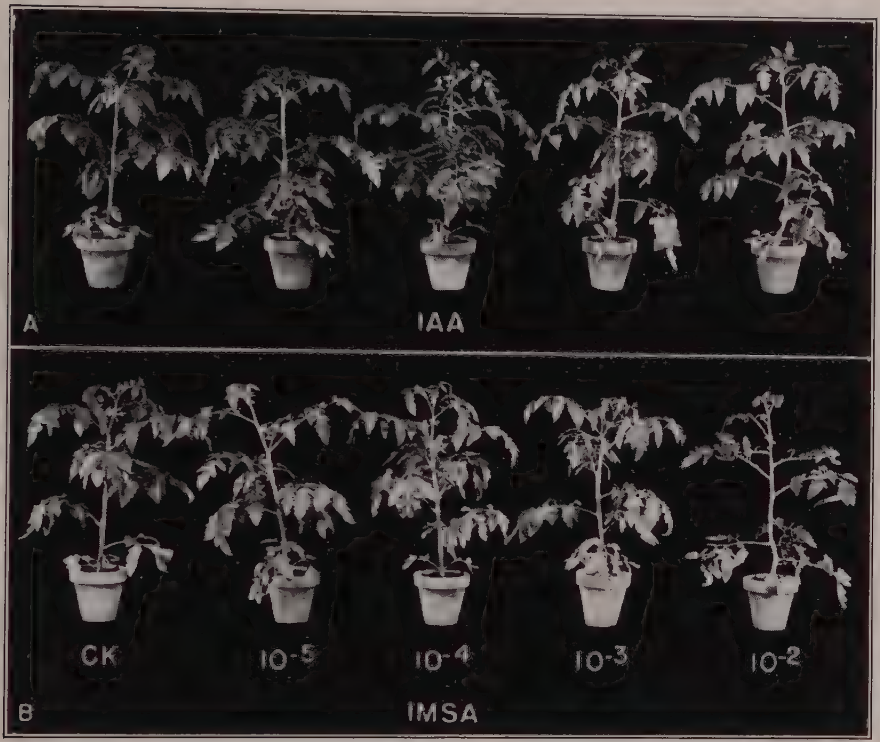

FIGURE 1. Tomato plants 14 days after spraying with aqueous solutions of growth regulants. (A) IAA at $10^{-5}M$ to $10^{-2}M$, and (B) IMSA at $10^{-5}M$ to $10^{-2}M$.

TABLE II

EFFECT OF SPRAYS OF IMSA ON ELONGATION OF TOMATO SEEDLINGS

Treatments	Concentration (parts per million)	Combined increase in height (cm.) of five replicate tomato seedlings after days indicated			
		4	8	11	14
IMSA	100	57.0	75.0	79.0	93.5
	250	66.5	83.5	89.5	103.0
	500	74.6	94.5*	100.5*	115.0*
	750	76.0*	93.0	101.5*	113.0
	1000	72.0	87.5	92.5	107.0
H_2O	—	59.5	74.0	78.5	91.0

* Significantly different from controls at 5 per cent level of confidence, based on system of allowances.

RESPONSE OF BEAN SEEDLINGS TO IMSA

Bean (*Phaseolus vulgaris* var. Tendergreen) seedlings were grown in composted soil until they were ten days old, at which time they were sprayed with solutions of IMSA and IAA at concentrations of $10^{-2}M$ to $10^{-5}M$. Stem curvature responses were recorded after 1, 3 and 9 days, while leaf modification and proliferation responses were noted 9 days after the initial application of treatments. Results are presented in Table III.

TABLE III

COMPARATIVE ACTIVITY OF IMSA AND IAA FOR INDUCING RESPONSES IN INTACT BEAN SEEDLINGS

Treatment applied		Response of seedlings*				
Material	Molarity	Stem curvature after			Leaf modification after 9 days	Proliferation after 9 days
		1 day	3 days	9 days		
H_2O	—	o	o	o	o	o
IMSA	10^{-2}	+	+	++	+++	+
	10^{-3}	o	o	o	++	o
	10^{-4}	o	o	o	+	o
	10^{-5}	o	o	o	o	o
IAA	10^{-2}	+++	++	o	o	o
	10^{-3}	++	+	o	o	o
	10^{-4}	+	o	o	o	o
	10^{-5}	o	o	o	o	o

* o = No response.
+ = 5° to 10° stem curvature; slight leaf modification; slight proliferation.
++ = 11° to 20° stem curvature; moderate leaf modification; moderate proliferation.
+++ = >20° stem curvature; severe leaf modification; severe proliferation.

IMSA at $10^{-2}M$ caused rapid stem curvatures to develop in bean seedlings. These curvatures persisted for at least 9 days following the initial treatment, while IAA at $10^{-2}M$ caused severe curvatures, but recovery from curvature was more rapid than in IMSA treatments. This result is consistent with the observations in the tomato assay. Leaf modification was again induced by IMSA at $10^{-2}M$ to $10^{-4}M$, while IAA was inactive in this respect. IMSA at $10^{-2}M$ also induced slight proliferation of stems whereas IAA at a similar dosage did not induce this response.

RESPONSE OF AVENA SECTIONS TO IMSA

The growth regulant action of IMSA was examined in the *Avena* first internode assay of Nitsch and Nitsch (5). This assay is a useful tool for measuring the effect of low concentrations of chemicals on cellular extension and, in some cases, on cellular division. *Avena* sections were prepared as previously described (5). Sections 4 mm. in length, of the first internode were immersed in 1.0 ml. phosphate-citrate buffer ($10^{-2}M$ K_2HPO_4 at pH 5.0) containing 2.0 per cent sucrose and $10^{-2}M$ to $10^{-6}M$ concentrations of IMSA and IAA. Growth of ten sections per treatment was recorded after 22 hours. The results are presented in histogram form in Figure 2.

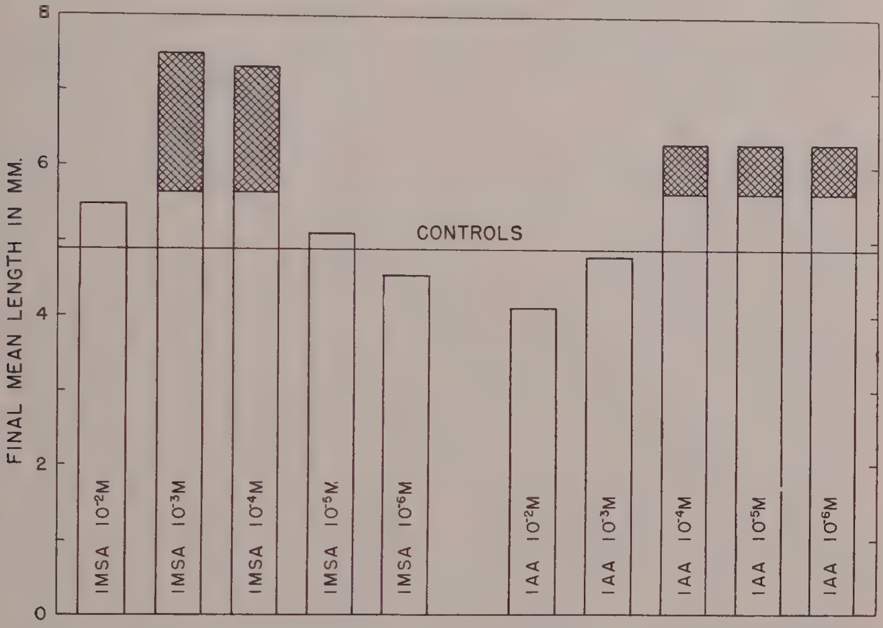

FIGURE 2. Effects of IAA and IMSA at different concentrations on the elongation of the first internode of *Avena* seedlings. (Solid areas represent statistically significant increases in elongation.)

IMSA promoted the growth of first internode sections immersed in $10^{-3}M$ and $10^{-4}M$ solutions of the chemical. IAA inhibited growth at $10^{-2}M$ but was stimulatory in the range of $10^{-4}M$ to $10^{-6}M$ (Fig. 3).

RESPONSE OF ETIOLATED PEA SECTIONS TO IMSA

Six-day-old pea (*Pisum sativum* L. var. Alaska) seedlings, grown in the dark, were prepared for use in the pea curvature assay following the procedure outlined by Leopold (4). Ten sections were floated in aqueous solutions of IMSA and IAA, used at $10^{-3}M$ to $10^{-6}M$ concentrations. Relative amounts of curvature were recorded after 4 and 30 hours. Results are given in Table IV.

Moderate to slight curvatures were induced by IMSA at $10^{-3}M$ and $10^{-4}M$ after 4 hours. After 30 hours IMSA at $10^{-3}M$ maintained moderate curvature of the sections. IAA caused from severe to slight curvatures in the range of $10^{-3}M$ to $10^{-6}M$ (Fig. 4).

TABLE IV

COMPARATIVE ACTIVITY OF IMSA AND IAA FOR INDUCING CURVATURE OF ETIOLATED SLIT PEA STEM SECTIONS

Treatment	Molarity of solution	Curvature after	
		4 Hours	30 Hours
Controls	—	0	0
IMSA	10^{-3}	3	3
	10^{-4}	1	0
	10^{-5}	0	0
	10^{-6}	0	0
IAA	10^{-3}	2	P
	10^{-4}	4	4
	10^{-5}	3	2
	10^{-6}	1	1

0 = No response; 1 = minimum curvature; 4 = maximum curvature; P = plasmolysis.

RELATIVE ACTIVITIES OF IMSA AND IAA FOR THE SUPPRESSION OF GROWTH OF CUCUMBER ROOTS

The influence of IMSA and IAA on the growth of roots of cucumber (*Cucumis sativus* L. var. Chicago pickling) seedlings was evaluated employing the method of Ready and Grant (6). Ten cucumber seeds were placed on filter paper in 9-cm. diameter Petri dishes and were treated with 5 ml. of H_2O or 5 ml. of aqueous solutions containing $10^{-4}M$ to $10^{-7}M$ IMSA and IAA. Triplicate series were included per treatment. The cultures were maintained at 25° C. for 3 days, then root lengths were measured. Results are presented in Table V. IMSA and IAA both suppressed root growth significantly at a concentration of 10^{-4} to $10^{-5}M$.

TABLE V

COMPARATIVE ACTIVITY OF IMSA AND IAA FOR THE SUPPRESSION OF GROWTH OF ROOTS OF CUCUMBER SEEDLINGS

Treatment	Molarity of solution	Average root length (mm.) for 30 seedlings
H_2O	—	69.4
IMSA	10^{-4}	16.3**
	10^{-5}	30.4**
	10^{-6}	74.9
	10^{-7}	59.6
IAA	10^{-4}	30.9**
	10^{-5}	39.0**
	10^{-6}	58.1
	10^{-7}	67.1

** Significantly different from controls at 1 per cent level of confidence, based on system of allowances.

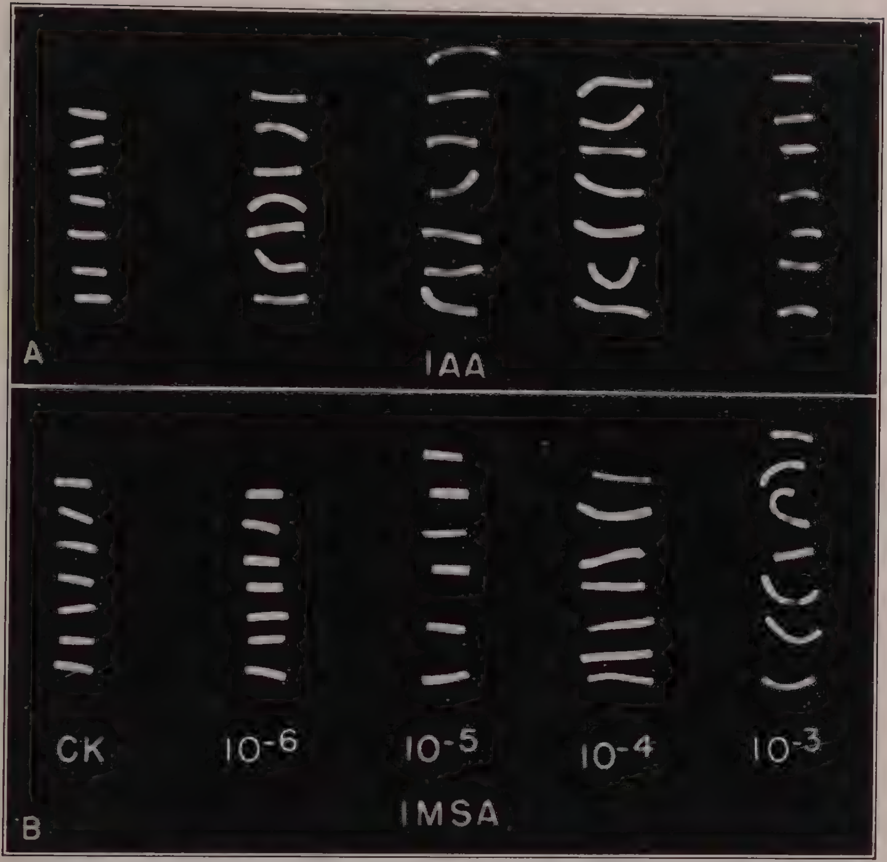

FIGURE 3. *Avena* first internode sections after exposure for 24 hours (from left to right) to (A) buffer control, IAA $10^{-6}M$ to $10^{-3}M$, and (B) buffer control, IMSA $10^{-6}M$ to $10^{-3}M$.

PRE-EMERGENCE AND POST-EMERGENCE WEED CONTROL EXPERIMENTS WITH IMSA

The broad activity of IMSA in a number of plant growth regulant assays coupled with the unusual residual action of the compound in tomato and bean seedlings suggested that IMSA might possess herbicidal activity. To examine this possibility several rows of broccoli (*Brassica oleracea* L. var. *italica* Plenck.), cabbage (*Brassica oleracea* L.), radish (*Raphanus sativus* L. var. Scarlet Globe), and mustard (*Brassica nigra* Koch) were planted in a composted sod soil in metal flats. A mixture of weed seed containing perennial rye grass (*Lolium perenne* L.), wild mustard (*Brassica arvensis* L.), pigweed (*Amaranthus retroflexus* L.), lamb's quarters (*Chenopodium album* L.), crabgrass (*Digitaria sanguinalis* L.), timothy (*Phleum pratense* L.) and millet (*Chaetochloa italica* L.) was distributed evenly over the surface of the soil containing the seeded crops. IMSA

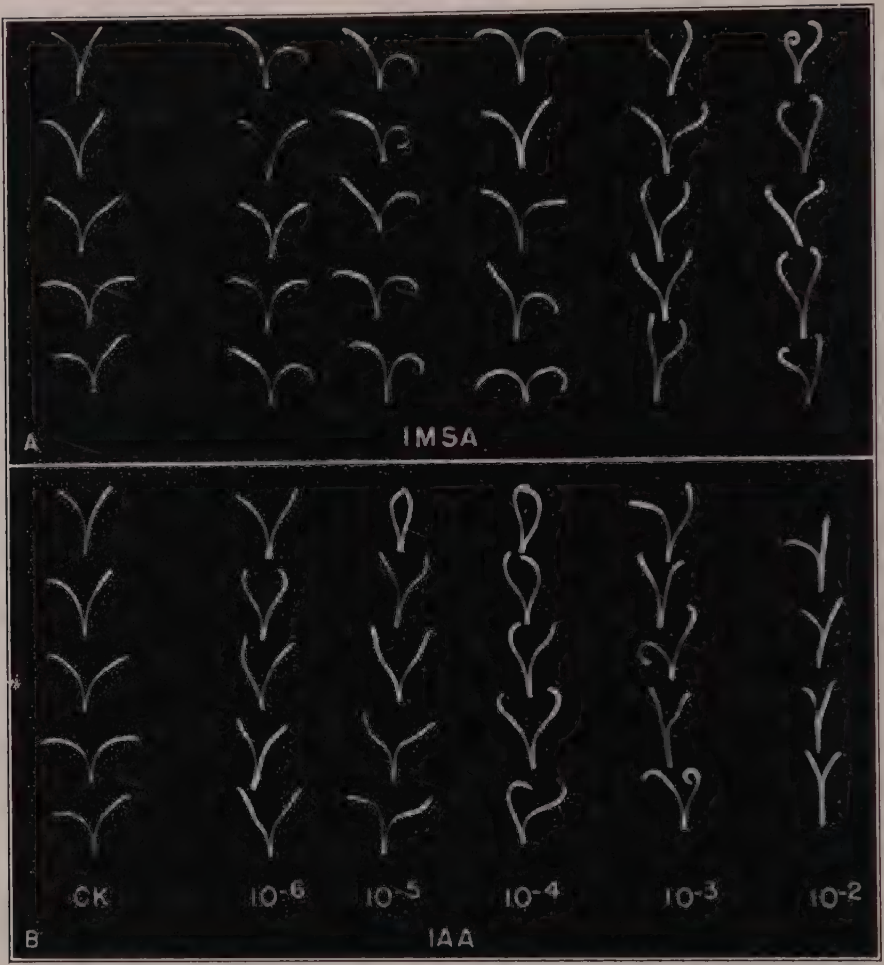

FIGURE 4. Etiolated pea sections after exposure (from left to right) to (A) H_2O, $10^{-6}M$ to $10^{-2}M$ IMSA; and (B) H_2O, $10^{-6}M$ to $10^{-2}M$ IAA.

was sprayed as a pre-emergence treatment, over the surface of the soil in an aqueous solution at the rate of 5, 10, and 20 lb. per acre. Controls were sprayed with water. Two weeks after treatment records were taken on crop tolerance and weed control (Table VI).

Slight control of both broad-leaved and grass weeds was noted at 5 lb. per acre (Fig. 5), moderate control at 10 lb. per acre, and excellent control at 20 lb. per acre. It is of interest to note the tolerance of radish to IMSA at dosages as high as 20 lb. per acre. Although IMSA was not compared directly with IAA in these experiments it has been generally noted[4] that

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[4] Personal communication from Dr. L. J. King, Boyce Thompson Institute for Plant Research, Inc., Yonkers, N. Y.

TABLE VI

Effect of IMSA on the Pre-Emergence Control of Weeds

Treatment	Lb./acre	Injury to crops				Weed control*	
		Broccoli	Cabbage	Radish	Mustard	Broad-leaved	Grasses
IMSA	5	None	None	None	Slight	2	2
	10	Slight	Slight	Slight	Moderate	3	3
	20	Severe	Severe	Slight	Severe	4	4
Controls	—	None	None	None	None	1	1

* Percentage weed control: 1 = 0–25 per cent, 2 = 26–50 per cent, 3 = 51–75 per cent, 4 = 76–100 per cent.

at least 15 to 20 lb. per acre of IAA must be applied to soil as a pre-emergence treatment to provide weed control equivalent to that obtained here with 5 to 10 lb. per acre of IMSA.

In post-emergence weed control experiments IMSA at 10 and 20 lb. per acre controlled grass and broad-leaved weeds in stands of oats, corn, and soybean (Table VII).

Oats tolerated 10 and 20 lb. per acre of IMSA applied as a post-emergence herbicide, while corn was injured slightly, and soybean injured severely by these rates of application. Control of broad-leaved and grass weeds was good at 10 lb. per acre of IMSA and excellent at 20 lb. per acre.

DISCUSSION

The most striking differences between the plant growth regulating activity of IMSA and other known indole growth regulators, such as IAA, are (a) the induction by IMSA of leaf modification and formative effects in tomato and bean; (b) the more persistent stem curvatures induced by IMSA and the slower recovery from symptoms; and (c) the tolerance of radish, and other members of the mustard family, as well as of established grasses to herbicidal dosages of IMSA.

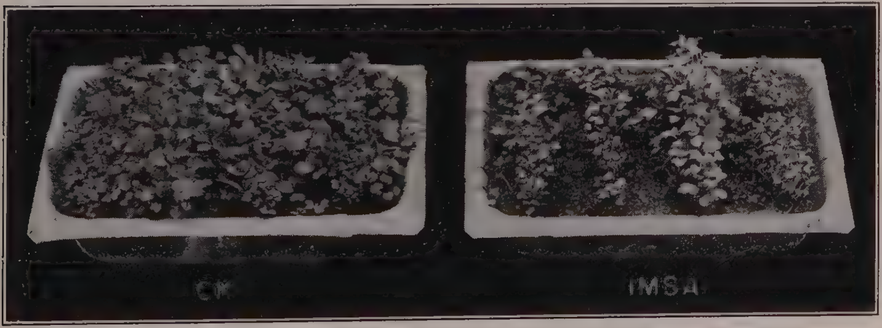

Figure 5. The control of broad-leaved and grass weeds with pre-emergence applications of 5 lb. per acre IMSA.

TABLE VII

EFFECT OF POST-EMERGENCE SPRAYS OF IMSA ON THE CONTROL OF WEEDS

Treatment	Lb./acre	Injury to crops			Weed control*	
		Soybean	Oat	Corn	Broad-leaved	Grasses
Controls	—	None	None	None	1	1
IMSA	10	Severe	None	Slight	3	3
	20	Severe	None	Slight	4	4

* Percentage weed control: 1 = 0–25 per cent, 2 = 26–50 per cent, 3 = 51–75 per cent, 4 = 76–100 per cent.

Although the plant growth regulating activity of certain indole compounds has been known for more than twenty years, this group of compounds has not been generally useful as herbicides. Two reasons have accounted for this. One has been the prohibitive cost of indole type growth regulators especially when greater than the amounts needed for hormone effects are used, and a second reason has been the rapid recovery from initial hormonal symptoms induced by compounds such as IAA. The rapid recovery from injury may be related to the capacity of plant tissues to metabolize IAA via oxidizing systems or to other systems of inactivation. The persistence of symptoms induced by IMSA in some tissues may be due to the lack in these tissues of systems capable of metabolizing the compound. Therefore its value as a selective herbicide would seem to be indicated.

The results presented here indicate that IMSA is an active plant growth regulator and probably is not an auxin antagonist as proposed by Wieland *et al.* (8). IMSA promotes the extension of *Avena* first internode sections, induces curvature of etiolated pea sections, and modifies the growth habit of both bean and tomato plants. In addition, the compound is active in suppressing the elongation of roots of cucumber seedlings. Based on the symptoms induced by IMSA in tomato seedlings it does not appear likely that the compound is converted to IAA before it can exert its growth regulating action, particularly since IMSA induces leaf modification in both tomato and bean while IAA does not. It follows therefore that, in addition to compounds possessing a ring system with unsaturation and with a side chain containing a carboxyl group (or a group readily converted to a carboxyl group), also such compounds with a sulfonic acid substituent in the side chain may be active plant growth regulators.

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CHEMISTRY AND CRYSTALLOGRAPHY OF SOME POLYBASIC CUPRIC CALCIUM SULFATES (BORDEAUX MIXTURES)[1]

Beatrice S. Magdoff, H. P. Burchfield, and Joan Schechtman

SUMMARY

A series of Bordeaux mixtures prepared with 1.00 to 3.38 moles of calcium hydroxide per mole of cupric sulfate was investigated by X-ray diffraction techniques and chemical methods of analysis. In general the concentration of cupric sulfate pentahydrate was maintained at 1.2 per cent (as in 10-10-100 Bordeaux mixture) since the products obtained varied with dilution. Rate studies carried out in dilute solution showed that fresh tribasic cupric sulfate bound 1 to 2 moles of lime rather slowly. However the hydrogel of 10-10-100 Bordeaux mixture (Cu:Ca=1:3.38) contained 2.5 atoms of calcium per 4 atoms of copper 5 minutes after mixing, but thereafter the rate of binding of calcium decreased. The average composition tended to approach 3 atoms of calcium per 4 atoms of copper for 10-6-100 Bordeaux (Cu:Ca=1:2.04) and 4 atoms of calcium per 4 atoms of copper for 10-10-100 Bordeaux. These compositions are not necessarily chemical compounds since X-ray evidence indicates that Bordeaux hydrogel contains at least two crystalline components which appear almost immediately after mixing. One of these probably consists of sizable crystallites (not less than 10^{-5} cm.), while the other can be indexed as a line broadened pattern of tribasic cupric sulfate of particle size about 50 Å.

The copper-containing end-products obtained by aging the mixtures under various conditions have been found to crystallize into four compounds. One of the compounds has been established previously as $Cu_5Ca_2SO_4(OH)_{12} \cdot H_2O$ and its unit cell determined (10). Two compounds crystallize into the orthorhombic system: a unit cell of dimensions $a=7.05$ Å, $b=14.10$ Å, $c=5.94$ Å with space group *Pmmn* containing 2 formula weights of $Cu_2Ca_2SO_4(OH)_6 \cdot 3H_2O$, and the other unit cell with $a=14.40$ Å, $b=20.90$ Å, $c=7.67$ Å containing 12 formula weights of $CuCa_2SO_4(OH)_4 \cdot H_2O$. The fourth, under some conditions of preparation, probably consists of more than one component, since analyses show that it may contain between 3 and 6 atoms of calcium per atom of copper. However, the X-ray powder pattern can be indexed on the basis of a hexagonal unit cell, where $a=7.56$ Å and $c=9.25$ Å, with one formula weight of $Cu_4Ca_4SO_4(OH)_{12} \cdot 3H_2O$.

INTRODUCTION

Bordeaux mixtures, obtained by mixing aqueous $CuSO_4$ with suspensions of $Ca(OH)_2$, have been used as fungicides for almost 75 years. Millardet and Gayon (13) postulated that the reaction products were calcium sulfate (gypsum) and cupric hydroxide. Later Bell and Taber (2) pointed out that the solid phases of some Bordeaux mixtures contained an excess of lime. Pickering (14) then showed that almost all of the cupric

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[1] The work described in this report was supported jointly by the United Fruit Co., Boston, Mass., the Phelps Dodge Refining Co., New York, N. Y., and the Standard Fruit and Steamship Co., New Orleans, La., from 1954 to 1957.

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ion was precipitated when 0.75 mole of lime was mixed with 1.00 mole of $CuSO_4$, and that the end-product was tribasic cupric sulfate, $Cu_4SO_4(OH)_6$. Sicard (16), Wöber (17), and later Martin (12) confirmed Pickering's findings.

The mixtures normally used as fungicides contain more than 0.75 mole of lime, which is the minimum necessary to precipitate all the copper. Pickering (14) ascribed a composition more basic than tribasic cupric sulfate to the products precipitated when somewhat larger amounts of lime were used. He found that the precipitate obtained on mixing 1.8 to 3.6 moles of lime per mole of $CuSO_4$ in dilute aqueous solution contained 5 atoms of copper, 2 of calcium and 1 sulfate radical. Recently, Fratini and Serra (10) found that the precipitates from dilute mixtures prepared with 1.6 to 3.5 moles of lime per mole of $CuSO_4$ crystallize in the hexagonal form, and that the composition of the compound is that determined by Pickering with the addition of 1 mole of water of hydration. However, the compounds which are formed in more concentrated mixtures such as 10-10-100 Bordeaux[2] and similar preparations containing smaller amounts of lime have not been identified although Baroni and Bettolo (1) have recorded their diffraction patterns.

This communication is concerned with the composition and properties of the materials that are formed on mixing 1.0 to 3.38 moles of lime with one mole of $CuSO_4$ (10-3-100 to 10-10-100 Bordeaux mixture) at concentrations normally employed in preparing fungicide sprays. It describes some of the properties of the hydrogel which is formed immediately on mixing the lime and cupric sulfate, and 4 chemical compounds, one of which was previously prepared by Pickering (14), and later characterized by Fratini and Serra (10).

MATERIALS AND METHODS

ANALYTICAL PROCEDURES

Calcium determination. A 0.01M solution of ethylenediaminetetraacetic acid (EDTA) was used for titrating calcium. The indicator was prepared by dissolving 0.2 gram of Eriochrome Black T in a mixture of 15 ml. of triethanolamine and 5 ml. of absolute ethanol. The buffer contained 67.5 grams of ammonium chloride dissolved in 570 ml. of concentrated ammonia diluted to 1 liter.

The copper-containing fraction of Bordeaux mixture (0.100 to 0.150 g.) was dissolved in *ca.* 2 ml. of 6N acetic acid and made up to a volume of 50 ml. The solution was buffered with 3 ml. of the ammonium chloride-ammonium hydroxide mixture and then *ca.* 0.4 g. of sodium cyanide was added to complex the copper. After mixing thoroughly, a few drops of the indicator were added and the resulting wine red solution titrated to a blue color with 0.01 M EDTA (7).

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[2] This notation refers to a mixture prepared with 10 lb. of $CuSO_4 \cdot 5H_2O$ and 10 lb. of $Ca(OH)_2$ per 100 gallons of water. The usual convention is to state the composition in terms of 100 gallons of mixture.

Hydroxyl determination. A 0.100-g. sample of Bordeaux mixture was dissolved in 50.0 ml. of standard 0.1 N sulfuric acid and the solution back-titrated with 0.1 N sodium hydroxide. Two inflection points were obtained. The difference between the number of equivalents of acid which had been added and the number of equivalents of base required to reach the first inflection point gave the total amount of hydroxyl bound in the complex. The difference between the first and second inflection points gave the amount of copper as tribasic cupric sulfate (11).

Confirmation of results. Calcium, copper and hydroxyl titrations, and the iodometric method for copper were used for the preliminary analysis of hydrogels and crystalline products. However, all results reported in this paper were confirmed by re-analysis at the Phelps Dodge Refining Co. laboratories through the courtesy of Dr. S. B. Tuwiner. The electrolytic method for copper, the gravimetric oxalate method for calcium, and the barium method for sulfate were used. The number of equivalents of hydroxyl were calculated by balancing the charges and the remainder was attributed to the moles of water of hydration.

Physical measurements. X-ray diffraction powder patterns were recorded with a Norelco diffractometer, using 1° divergence and receiving slits, and a 0.003 inch scatter slit. A nickel filter, thick enough to eliminate 98 per cent of the Cu $K\beta$ radiation, was used with the Philips Cu target X-ray tube. The intensities of the patterns were weak, as can be noted by the high statistical fluctuations on the patterns reproduced here, which necessitated using a scale factor of 4 or 8 and a time constant of 2 seconds. When single crystals were available, the data were recorded on film using a Weissenberg camera. The cell dimensions and space groups were determined from oscillation and Weissenberg diagrams.

Density measurements were made at 25° C. in a 2-ml. pycnometer using water as a fluid.

BINDING OF LIME BY HYDROGELS

To avoid carbonation, studies on the binding of lime by hydrogels were made in a covered battery jar which contained sodium hydroxide pellets. A magnetic stirrer was used to keep the mixture in suspension. Reagents were introduced by pipettes through holes in the cover which were closed by rubber stoppers when not in use. The cover was sealed by applying a thick layer of stopcock grease to the rim of the jar.

Tribasic cupric sulfate was prepared by placing 150 ml. of a solution containing 3.60 g. of cupric sulfate pentahydrate in a beaker and adding 0.75 molecular equivalent of calcium hydroxide in 125 ml. of water. The binding of the first mole of lime per mole of tribasic cupric sulfate was studied by adding 0.27 g. in 25 ml. of water to the suspension 1 to 3 minutes after preparation and measuring the change in pH as a function of time with a Model G Beckman pH meter. The binding of the second mole of lime was studied in an independent experiment in which 0.27 g. of calcium hydroxide in 25 ml. of water was added 37.5 minutes after the addition of the first mole.

The rate of binding of lime by a 10-10-100 Bordeaux hydrogel (3.38 moles of lime per mole of cupric sulfate) was investigated at 5° C. by shaking 200-ml. aliquots of the hydrogel aged for various periods with 1700 ml. of cold water for 5 minutes and then centrifuging at 5° C. The precipitates were then analyzed for copper and calcium by the methods described above.

The amount of lime bound per atom of copper was determined by aging 10-6-100 and 10-10-100 mixtures for 24 hours at 5° C. and then extracting with various volumes of ice water. The suspensions were filtered, dried with alcohol and ether, and the precipitates analyzed for calcium and copper as described above.

PREPARATION OF MIXTURES FOR CRYSTALLIZATION

Method I. Bordeaux mixtures containing 10 lb. of $CuSO_4 \cdot 5H_2O$ per 100 gallons of water and from 3.0 to 10 lb. of lime were prepared as described previously (4, 5). Thus the initial compositions varied between 1.00 and 3.38 atoms of calcium per atom of copper. These materials were aged for 24 hours, 48 hours, and for various periods up to 2 years. A 10-10-100 mixture was prepared in a similar way and then aged for 40 days in a steam bath. This latter sample is hereafter referred to as heat-aged Bordeaux.

Method II. The effects of drying and re-wetting on Bordeaux mixtures prepared at low temperature were studied by preparing a 10-10-100 mixture (Cu:Ca = 1:3.38) at 5° C., and aging it at this temperature for 24 hours. It was then filtered on a Buchner funnel and resuspended in the same amount of water used in the initial preparation. A 10-6-100 mixture (Cu:Ca = 1:2.04) was prepared in a similar way except that it was aged for 8 days at 5° C. before filtration.

Method III. Six mixtures were prepared at standard concentrations using 1.00 to 2.25 moles of lime per mole of cupric sulfate in steps of 0.25 mole. They were aged on a shaking machine for 2.25 months at 10° C., and then at room temperature for 5 days.

Method IV. Two series of Bordeaux mixture containing 1.35 and 3.38 moles of lime per mole of cupric sulfate were prepared in the usual way except that part of the cupric sulfate was replaced by cupric acetate. Each series had ratios of copper to sulfate of 1 to 0.125, 0.188, 0.250, 0.500, and 0.750. These were aged at room temperature for 2 days.

ISOLATION OF COPPER-CONTAINING FRACTIONS

The copper-containing fractions of the Bordeaux mixtures described above were separated from gypsum ($CaSO_4 \cdot 2H_2O$) and lime by permitting the aqueous suspensions to settle and removing the top layer of sediment with a glass tube attached through a filter flask to a vacuum line. Much of the lime and gypsum was removed by this process since they tended to accumulate in the upper layer while the copper fractions of many Bordeaux mixtures tended to form compact sediments at the bottom of the container. The lower layers were then washed with successive portions of water until particles of lime and gypsum could no longer be detected under the microscope. In general about 500 to 1,000 ml. of wash water per gram of Bordeaux solids were required to obtain materials of satisfactory purity. It was necessary to avoid prolonged contact of the suspensions with air to prevent the formation of calcium carbonate which could not be washed out. After the lime and gypsum were removed, the suspensions were filtered, the copper fractions washed with alcohol and ether, and then dried in an oven for 5 hours at 110° C.

EXPERIMENTAL RESULTS AND DISCUSSION

BORDEAUX HYDROGELS

Hydrogel formation. The compound obtained by precipitating one mole of cupric sulfate with 0.75 mole of calcium hydroxide is tribasic cupric sulfate [$Cu_4SO_4(OH)_6$] (14). The addition of more lime results in the binding or adsorption, or both, of calcium and hydroxyl groups into the tribasic gel. The amount of lime lost from the supernatant and incorporated into the hydrogel depends upon concentration and reaction time.

The rate of binding of two equivalents of hydroxyl (one mole of lime) to freshly precipitated tribasic cupric sulfate was measured by taking pH readings as a function of time. pH values provide an approximate idea of the amount of hydroxyl bound but they can not be interpreted literally since the relation between pH and hydrogen ion concentration is not exact. The initial pH was about 0.5 unit lower than a suspension of free lime at equivalent concentration which suggests that about one-half of the hydroxyl ion was bound instantaneously. However, this may not represent true binding since charged colloids often produce aberrations in pH readings (9). After the initial incorporation of hydroxyl groups a short lag period occurred followed by an increased rate of binding and finally by a decreased rate as the concentration of hydroxyl ion in solution diminished (Fig. 1). Since Rich (15) has shown that Bordeaux mixture is positively charged it is evident that calcium ions also must have been bound to the hydrogel. Thus within an hour after mixing, the tribasic cupric sulfate bound or reacted with most of the lime that was added. In an independent experiment a second mole of lime was added 37.5 minutes after the first mole. Again the initial pH was lower than for a lime suspension of equivalent concentration, and it dropped rapidly indicating that a considerable portion of the second mole was also bound before the system appeared to reach equilibrium.

Since the pH changes that would occur on adding 3 or 4 moles of lime would be too small to measure, binding in this region was studied at a much higher concentration of reactants by preparing a 10-10-100 Bordeaux mixture (3.38 atoms of calcium per atom of copper). These experiments were carried out at 5° C. to avoid spherulite[3] formation (5). The copper-containing hydrogel was isolated at various time intervals after mixing by extracting the gypsum and unreacted lime with water. It was then dried and analyzed for calcium and copper. It was found that about 2.5 atoms of calcium per 4 atoms of copper were bound very rapidly under these conditions while an additional mole was bound slowly (Fig. 2).

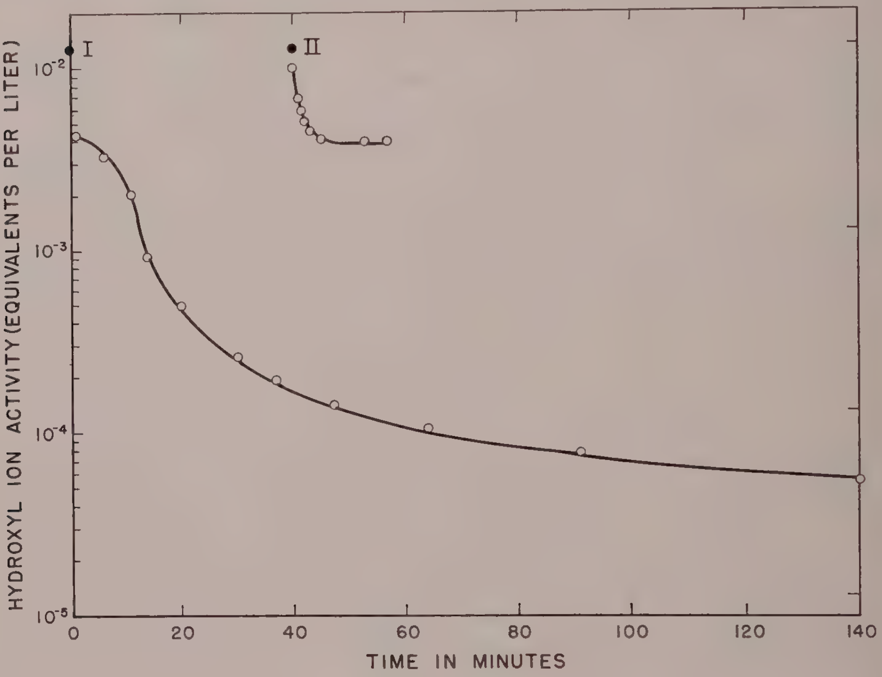

FIGURE 1. I. Binding of hydroxyl ion by a molecular equivalent of freshly precipitated tribasic cupric sulfate as measured by the decrease in pH of the mixture with time. II. Rate of binding of a second mole of lime added to a duplicate sample 37.5 minutes after the first mole. The black dot shows the pH of a solution of lime of equivalent concentration.

Composition of hydrogels. To determine the ultimate compositions of

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[3] The word spherulite is used to indicate the sphaerocrystals mentioned in earlier publications since it has wider acceptance.

10-10-100 and 10-6-100 Bordeaux hydrogels, they were prepared and stored at 5° C. for 24 hours which should be long enough for the reaction to approach completion. At the same time the formation of spherulites is suppressed. Aliquots of the mixtures were then extracted with increasing volumes of ice water. The number of atoms of calcium per atom of copper remaining in the solid phase decreased rapidly as unreacted calcium hydroxide and calcium sulfate were leached out (Fig. 3) until a limiting value was reached. Thereafter the rate of removal of calcium became very slow. Presumably, the remaining calcium was bound firmly in the hydrogels since it failed to dissolve readily in the water. The chemical compositions of the residues after all of the unbound lime and calcium sulfate were leached out were $Cu_4Ca_{2.98}(SO_4)_{1.23}(OH)_{11.50}$ for the 10-6-100 mixture and $Cu_4Ca_{3.87}(SO_4)_{1.58}(OH)_{12.58}$ for the 10-10-100 mixture. These empirical formulae were calculated using 4 atoms of copper as a base since the spherulites which are ultimately obtained from Bordeaux mixtures in this composition range usually contain 4 atoms of copper per sulfate radical. It should be noted that these are not compositions of homogeneous systems for, as will be shown below, more than one crystalline component is present very soon after mixing lime with cupric sulfate.

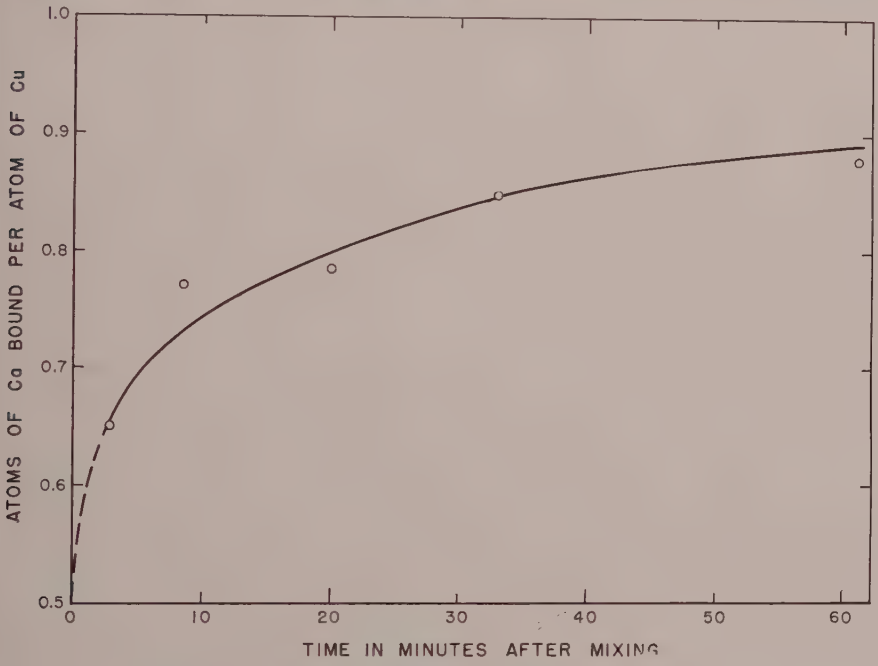

FIGURE 2. Binding of calcium by a hydrogel prepared with 3.38 moles of calcium hydroxide per mole of cupric sulfate (10-10-100 Bordeaux mixture).

Structure of a hydrogel. X-ray diffraction patterns were taken of the copper-containing fractions after aging mixtures prepared with 3.38 moles of lime per mole of cupric sulfate for 3 to 60 minutes at 5° C. The material was washed to remove lime and gypsum and then dried with alcohol and ether to eliminate any extraneous peaks and background in the patterns. When the pattern of the wet precipitate which had been aged for 3 minutes at room temperature was recorded it was the same except for the relative intensities of the peaks. The pattern (Fig. 4) of the material aged for 3 minutes shows that the hydrogel contains crystalline components within a few minutes after mixing, although it remains in suspension for about 5 hours at room temperature, and for several days at 5° C. This is similar to the immediate crystallization obtained with basic cupric nitrate and chloride (8). Two kinds of peaks are present; broad ones arising from small particles and sharp peaks which are Bragg reflections from crystallites with at least 1 dimension of about 10^{-5} cm. As the material is aged the Bragg reflections become sharper and more intense at the expense of the broad bands.

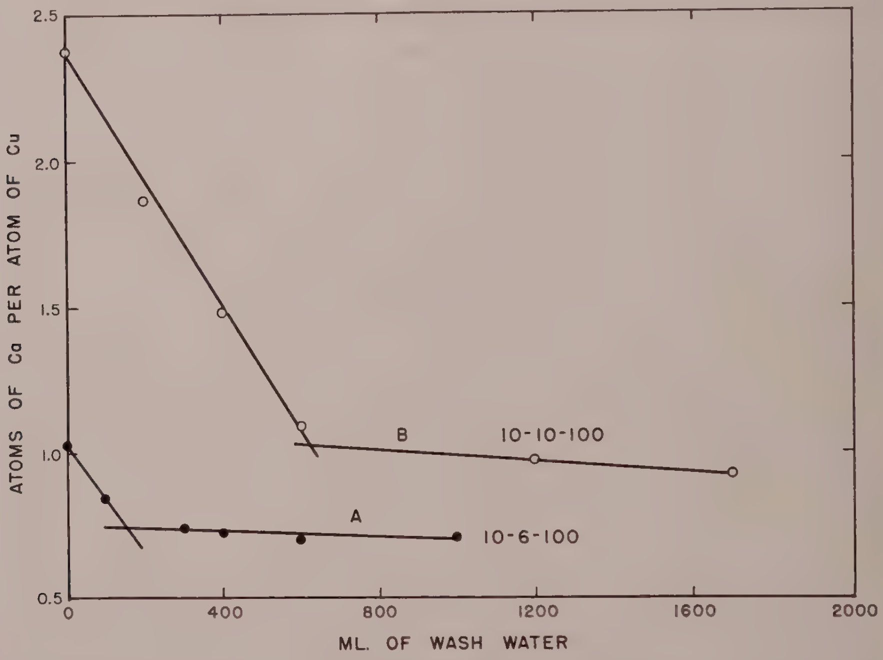

FIGURE 3. Effect of volume of wash water on the amount of calcium retained in solid phases prepared from (A) 2.04 and (B) 3.38 moles of calcium hydroxide per mole of cupric sulfate.

For convenience, the broad bands have been abstracted from the rest

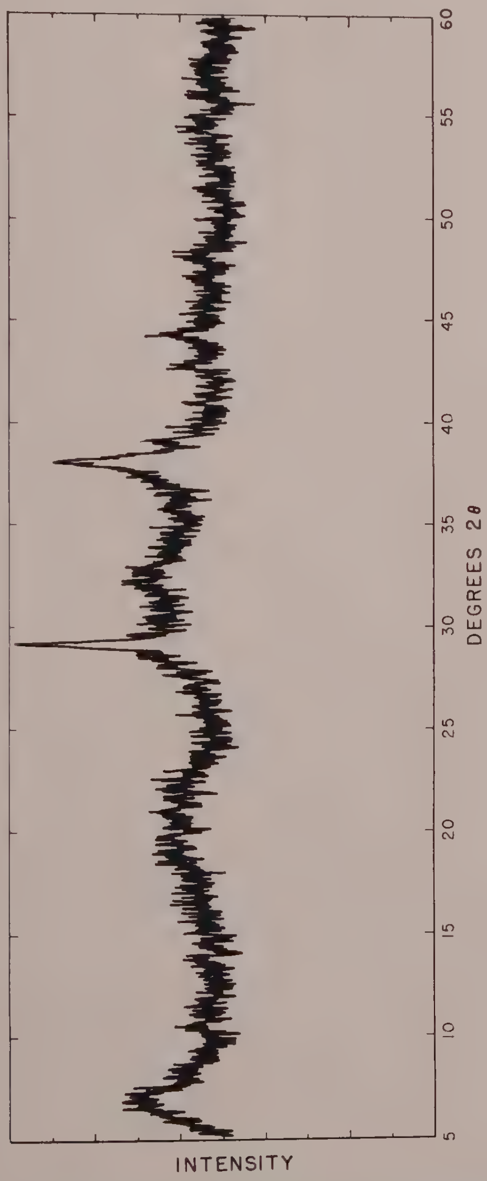

FIGURE 4. X-ray diffraction pattern of a hydrogel prepared from 3.38 moles of calcium hydroxide per mole of cupric sulfate (10-10-100 Bordeaux) and aged for 3 minutes at 5° C.

of the pattern and the relative $|F|^2$ plotted as a function of reciprocal spacing (Fig. 5). The broad bands with reciprocal spacings above 0.15 Å^{-1} correspond to the diffraction pattern of tribasic cupric sulfate [$Cu_4SO_4(OH)_6$] with particle size of 50 to 75 Å. It should be noted that this correspondence does not constitute an unequivocal identification since each band consists of more than one Bragg reflection. However, the similarity between these patterns and a calculated line-broadened pattern of tribasic cupric sulfate with particle size of 50 to 75 Å is striking. The comparison pattern used for calculation was obtained by preparing the crystalline powder according to Pickering (14) and by checking the A.S.T.M. catalogue #2-0299 (6) as well. With aging, both the particle size and the amount of this component diminishes, as is seen by a broadening of the bands and a decrease in intensity.

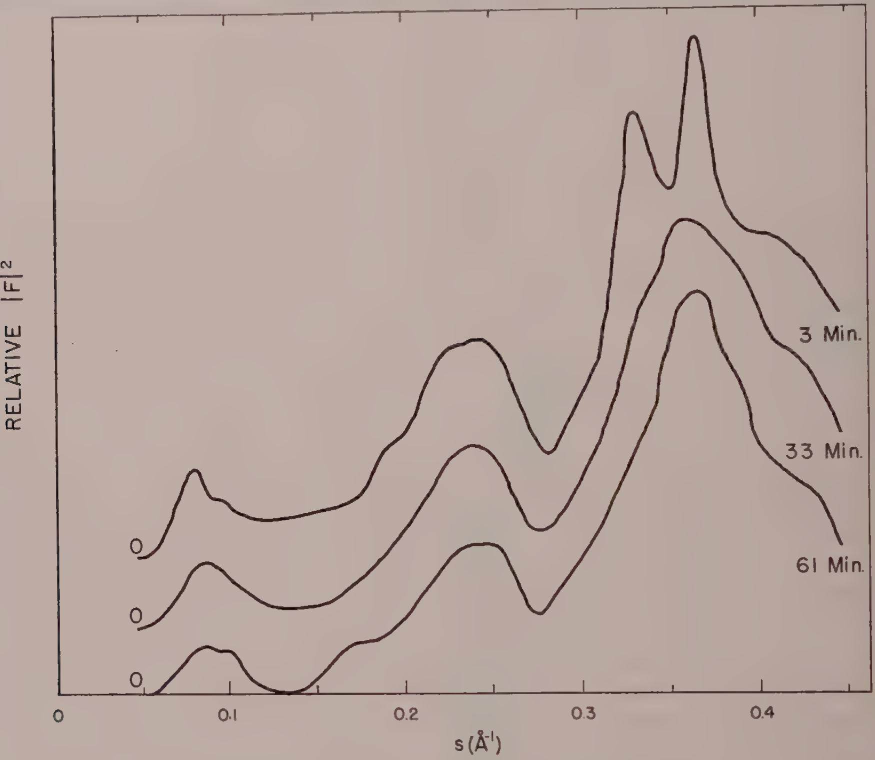

FIGURE 5. Corrected intensities, on an arbitrary scale, plotted against reciprocal spacings in Å^{-1} of the broad peaks from the X-ray diffraction patterns of 10-10-100 hydrogels aged at 5° C. for 3, 33 and 61 minutes. The arbitrary zero for each curve is indicated at its left.

The broad band at $s=0.078$ Å^{-1} which is present after aging for 3 minutes shifts towards larger reciprocal spacing within 33 minutes (Fig. 5). This is indicative of particle growth, and can be associated with the observed sharpening of the Bragg reflections upon aging. Platelets of relatively large diameter with a thickness of 50 to 100 Å would give such a pattern. Thus, the freshly prepared hydrogel probably consists partly of tribasic cupric sulfate and platelets. Upon further aging, pattern B (Fig. 8) is obtained. Because of the diminution of the particle size of tribasic cupric sulfate or its transformation into another compound with aging, the broad bands are either obscured or have disappeared as observed in pattern B.

CRYSTALLINE PRODUCTS

The amount of lime used in the initial preparation and the conditions of aging determine which of the four crystalline end-products is obtained. A phase contrast microscope was used to observe the process of crystallization since under ordinary illumination the hydrogel is transparent and only particles of lime are visible. With phase contrast the lime appears imbedded in flocs of precipitated hydrogel (Fig. 6 A). After aging for 4.5 hours at 22° to 26° C., the flocs condense somewhat (regions a, b, and c, Fig. 6 B) and gypsum crystals start to appear in region c. In the same field, 5.5 hours after preparation of the mixture (Fig. 6 C), the gypsum crystals have grown to considerable size and the hydrogel has crystallized into small spheroids about 6 μ in diameter. The spherulites, shown under higher magnification (Fig. 6 D) with the lime and gypsum washed out, are typical of compound B described below.

Pattern A. The crystallites giving this pattern (Fig. 7) were described by Fratini and Serra (10), who found that they were formed in Bordeaux mixtures prepared with 1.6 to 3.5 moles of lime per mole of cupric sulfate. However, these authors used a saturated solution of lime water in preparing their mixtures so they were always much more dilute than those commonly used as fungicide sprays. At the concentrations used in these investigations this compound occurs only within a range of 1.0 to 1.6 moles of lime per mole of cupric sulfate.

When prepared in dilute solution (10) the composition of this material is $Cu_6Ca_2SO_4(OH)_{12} \cdot H_2O$, but in mixtures based on 1.2 per cent concentration of aqueous $CuSO_4 \cdot 5H_2O$ at least one other phase is present. Thus two of the samples analyzed (Table I) are too low in calcium and too high in sulfate, while another is too high in both calcium and sulfate. One of the samples in this composition range agrees very closely with the compound described by Fratini and Serra (10) except that the water of hydration is too high.

In concentrated Bordeaux mixtures containing equimolar amounts of lime and cupric sulfate the crystallites decompose to yield CuO on aging

(5), but are stable when the lime content is increased to 1.07 moles. The particle size of these latter crystallites is about 100 Å as can be seen from the line broadening of the reflections (Fig. 7 A). These particles will remain hydrated indefinitely without further crystal growth, but once filtered and dried they can not be re-hydrated by suspending them in water. Mixtures prepared with 1.35 moles of lime give larger crystallites (Fig. 7 B). The X-ray patterns were sharper and better resolved when the crystallites had been prepared by method III.

The primitive unit cell is hexagonal, with dimensions $a = 11.80$ Å and $c = 14.31$ Å, and contains 2 formula weights of $Cu_5Ca_2SO_4(OH)_{12} \cdot H_2O$. Insufficient reflections were observed to determine the space group.

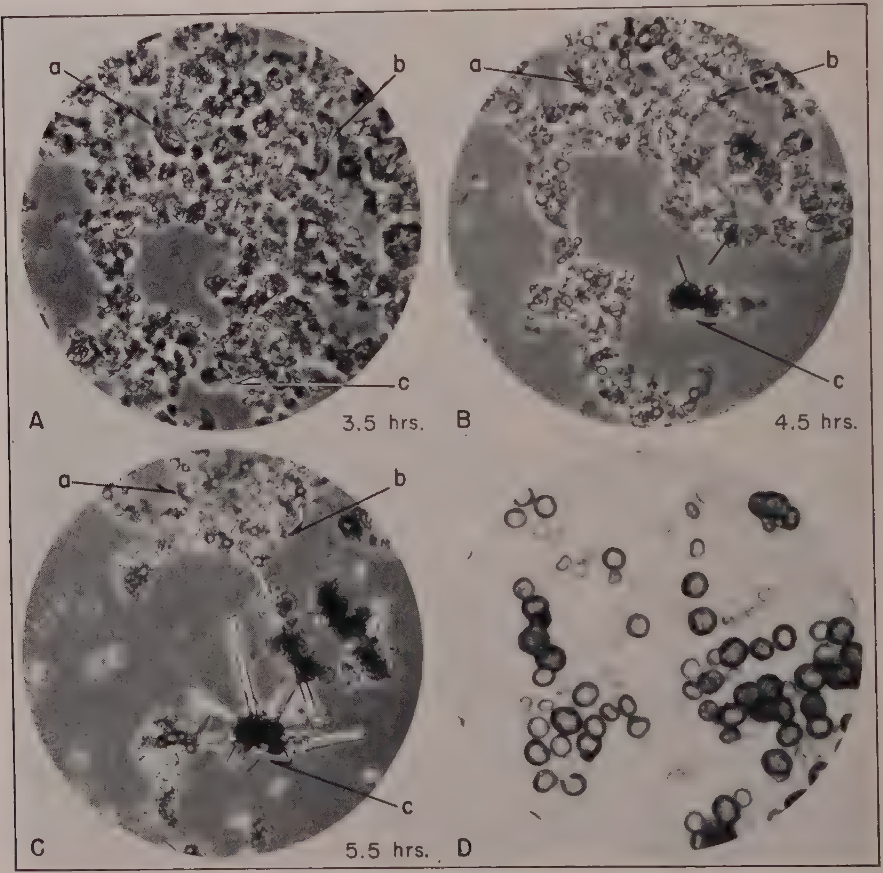

FIGURE 6. (A) 10-10-100 Bordeaux mixture aged for 3.5 hours at 22° to 26° C. and photographed under phase contrast illumination (×180). (B) The same field one hour later. (C) The same field two hours later. (D) Spherulites isolated from 10-10-100 Bordeaux aged 24 hours (×570).

TABLE I

COMPOSITIONS OF BORDEAUX CRYSTALLITES, COMPOUND A

Initial composition moles of lime per mole of cupric sulfate	Method of crystallization	Aging, da.	Elemental composition					Apparent density, g./ml.
			Cu	Ca	SO_4	OH	H_2O	
1.07	I	18	5.00	1.25	1.10	10.30	1.50	—
1.35	I	120	5.00	1.60	1.10	11.00	2.16	2.99
1.25	III	72	5.00	1.98	1.09	11.74	1.63	—
2.04	II	21	5.00	2.25	1.25	12.00	4.25	2.77

Fratini and Serra (10) obtained the same dimensions, but the indexing of the pattern shows marked differences (Table II).

Pattern B. Bordeaux mixtures prepared with 1.70 to 3.38 moles of lime per mole of cupric sulfate by method I form spherulites on aging. They are birefringent under the polarizing microscope. Detailed studies were limited to mixtures containing 3.38 moles of lime or less since a preparation made with as much as 6.8 moles of lime gives the same X-ray diffraction pattern indicating that compound B is formed over a very wide range of initial compositions. The X-ray diffraction patterns (Fig. 8 A and B) can be indexed (Table III) on the basis of hexagonal cell, with $a=7.56$ Å and $c=9.25$ Å, which contains one formula weight of $Cu_4Ca_4SO_4(OH)_{14}\cdot 3H_2O$.

The chemical compositions and apparent densities of crystallites giving pattern B prepared by different methods and aged for various times are

TABLE II

INDEXING OF PATTERN A

d (in Å)	$\sin^2\theta\times 10^2$		*hkl*
	Obs.	Calc.	
10.04	0.589	0.567	100*
7.22	1.164	1.137	002
4.55	2.865	2.868	112*
3.57	4.656	4.655	004
3.25	5.319	5.617	301*
3.09	6.267	6.210	302
3.03	6.588	6.465	213†
2.84	7.371	7.368	310
2.74	7.970	7.906	222, 115†
2.51	9.543	9.416	205†
2.42	10.236	10.131	402
2.35	10.773	10.739	320
2.03	14.259	14.252	007*
2.00	14.826	14.830	107*
1.74	19.549	19.589	406*
1.70	20.412	20.526	600*

* *hkl* assignment differs from Fratini and Serra (10).
† Not observed by Fratini and Serra (10).

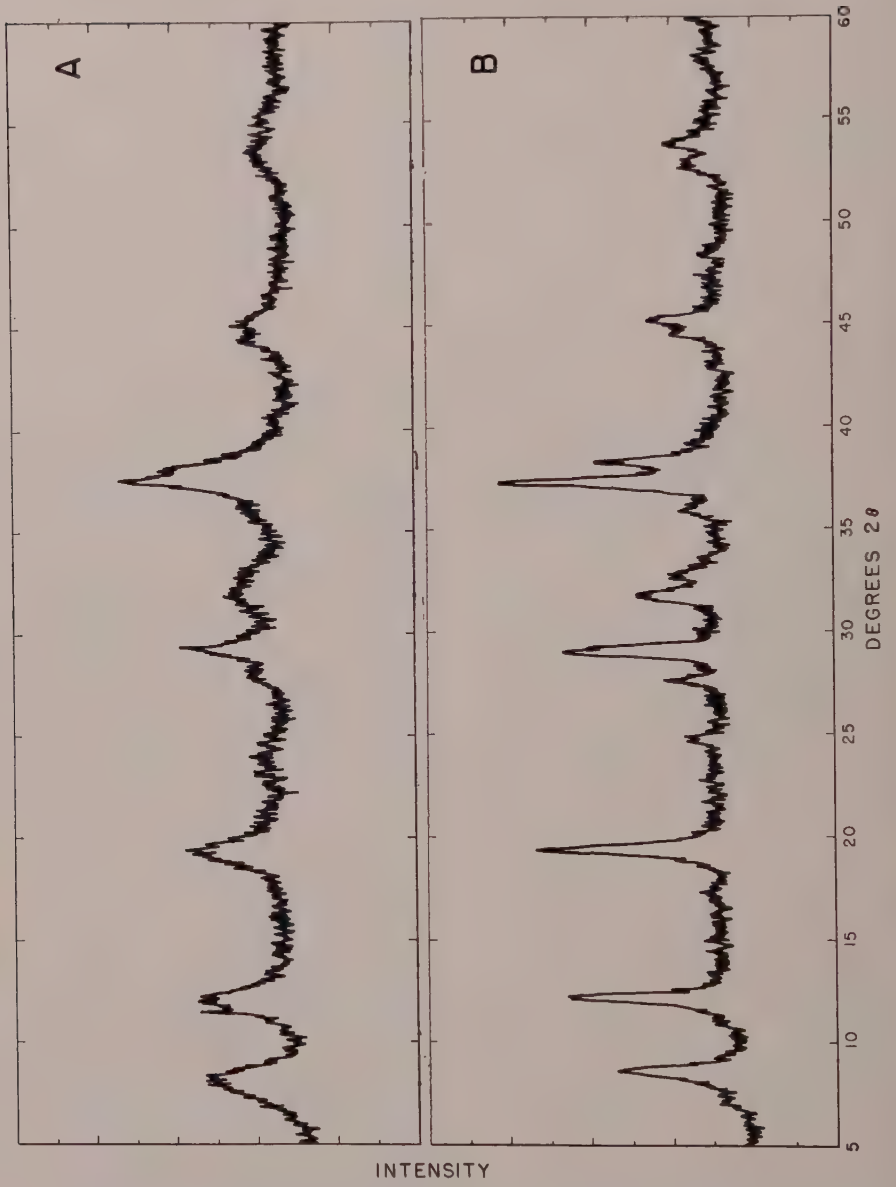

FIGURE 7. X-ray diffraction patterns of compound A obtained from the copper fractions of aged Bordeaux mixtures prepared with different amounts of calcium hydroxide. (A) 1.07 moles calcium hydroxide per mole of cupric sulfate (10-3.2-100 Bordeaux). (B) 1.35 moles calcium hydroxide per mole of cupric sulfate (10-4-100 Bordeaux).

TABLE III

INDEXING OF PATTERN B

d (in Å)	$\sin^2 \theta \times 10^2$		hkl
	Obs.	Calc.	
9.25	0.692	0.692	001
6.55	1.382	1.368	200
4.55	2.868	2.768	002
3.37	5.222	5.162	212
3.29	5.481	5.472	400
3.20	5.796	5.846	302
3.12	6.093	6.164	401
3.03	6.465	6.498	320
2.75	7.840	7.874	411
2.65	8.451	8.550	500
2.44	9.953	9.950	412
2.35	10.739	10.674	313
2.29	11.308	11.318	502
2.25	11.702	11.700	403
2.10	13.396	13.411	413
2.02	14.521	14.716	610
1.92	16.092	16.106	522
1.87	16.934	16.858	700
1.77	18.943	18.881	433
1.73	19.826	19.626	702
1.69	20.781	20.862	710

given in Table IV. Ten samples aged for different periods of time (not listed) have a composition consistent with the hexagonal unit cell. The variations in the compositions of the other samples may be due to the presence of additional copper-containing components which could not be leached out by water. For example one of these samples is too low in calcium (Table IV) while others are too high. Hence, the observed densities may be average values.

On preparing Bordeaux mixtures by method II small crystals are obtained instead of spherulites. These give the same general X-ray patterns

TABLE IV

COMPOSITIONS OF BORDEAUX CRYSTALLITES, COMPOUND B

Initial composition moles of lime per mole of cupric sulfate	Method of crystallization	Aging, da.	Elemental composition					Apparent density, g./ml.
			Cu	Ca	SO_4	OH	H_2O	
3.38	I	2	4.00	3.08	0.85	12.48	3.28	—
3.38	I (2% Marasperse)	5	4.00	4.04	1.08	13.92	3.38	2.86
2.70	I	730	4.00	4.08	1.04	14.08	2.96	2.78
3.38	II	20	4.00	5.00	0.92	16.16	4.20	2.82
		60	4.00	5.44	0.87	17.15	4.68	—
		110	4.00	5.82	1.00	17.64	4.88	—

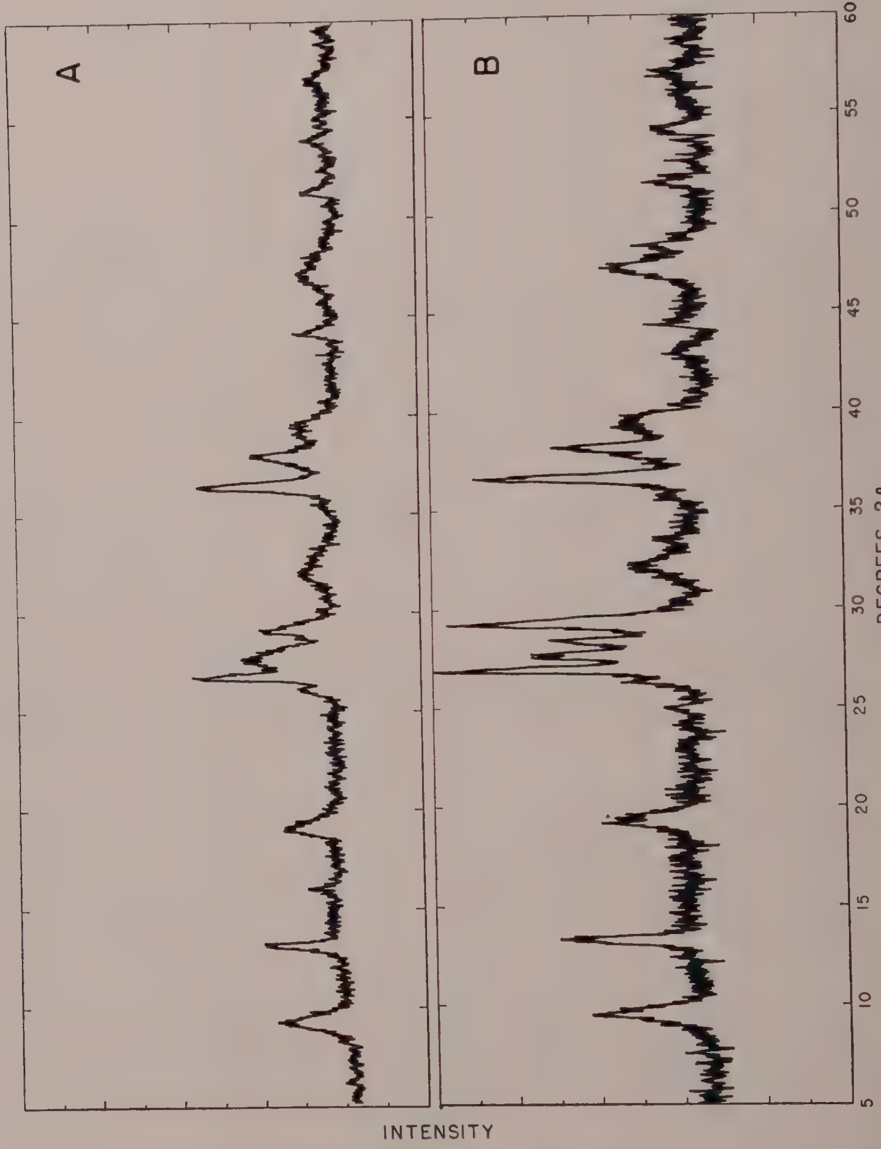

FIGURE 8. X-ray diffraction patterns of compound B crystallized from a mixture containing 3.38 moles of calcium hydroxide per mole of cupric sulfate (10-10-100 Bordeaux) by different methods. (A) Method I, giving typical spherulites; and (B) method II.

(Fig. 8 B) as the spherulites with some notable exceptions. Most important is the fact that the peaks no longer tend to tail off in intensity with increasing Bragg angle as in the pattern of the spherulites (Fig. 8 A). In addition, the resolution is improved, particularly the region of $2\theta = 28°$, and in general the peaks are sharper and more intense. Hence, the asymmetry of the peaks (Fig. 8 A) seems not to arise from a disordered layer structure, but rather from poor resolution due to very thin plate-like crystallites which pack radially in the spherulites. Such changes in the powder patterns suggest that the platelets grow thicker when not involved in spherulite formation.

Crystallites giving similar X-ray patterns can be obtained by replacing part of the cupric sulfate with cupric acetate (method IV). Even with an initial composition of 1.35 moles of lime per gram atom of copper the compound associated with pattern B was formed. Usually, this composition yields $Cu_5Ca_2SO_4(OH)_{12} \cdot H_2O$ as the major component. Since calcium acetate is considerably more soluble in water than calcium sulfate a higher concentration of calcium ion is present in solution which apparently results in faster binding of the lime by the hydrogel. Hence relatively thick platelets of $Ca_4Cu_4SO_4(OH)_{14} \cdot 3H_2O$ are formed.

Pattern C. Crystals with X-ray pattern C (Fig. 9 A) were obtained by aging Bordeaux mixtures prepared by method I which contained 1.70 to 2.36 moles of lime per mole of cupric sulfate. After about 3 months at room temperature some needle-like crystals of maximum size $80 \times 20 \times 5\ \mu$ appeared but spherulites still predominated, and the X-ray diffraction pattern was typical of compound B. With further aging more single crystals appeared, and by the end of two years spherulites were no longer present. The single crystals showed strong dichroism, suggesting a layered structure. This material gives diffraction pattern C.

All of the mixtures prepared by method III formed compound C only, except the one containing the smallest amount of lime. In this case compound A was present as well. With this method of preparation, the crystallites were extremely small and appeared to be aggregates. The formation of compound B is apparently by-passed at low temperatures, and compound C crystallizes slowly. This latter material has an empirical composition of $Cu_2Ca_2SO_4(OH)_6 \cdot 3H_2O$.

The cell dimensions, as determined from oscillation and Weissenberg diagrams are $a = 7.05$ Å, $b = 14.10$ Å, and $c = 5.94$ Å, giving a volume of 592 Å^3. Calculations using the formula weight, density (Table V) and the cell volume show that there are 2.03 "molecules" per cell, the combined error of all of these measurements being about 1.5 per cent. The space group is *Pmmn*, with systematic absences hOO, OkO and hkO when h, k and $h+k$ are odd, respectively.

Pattern D. The compound with X-ray pattern D (Fig. 9 B) was ob-

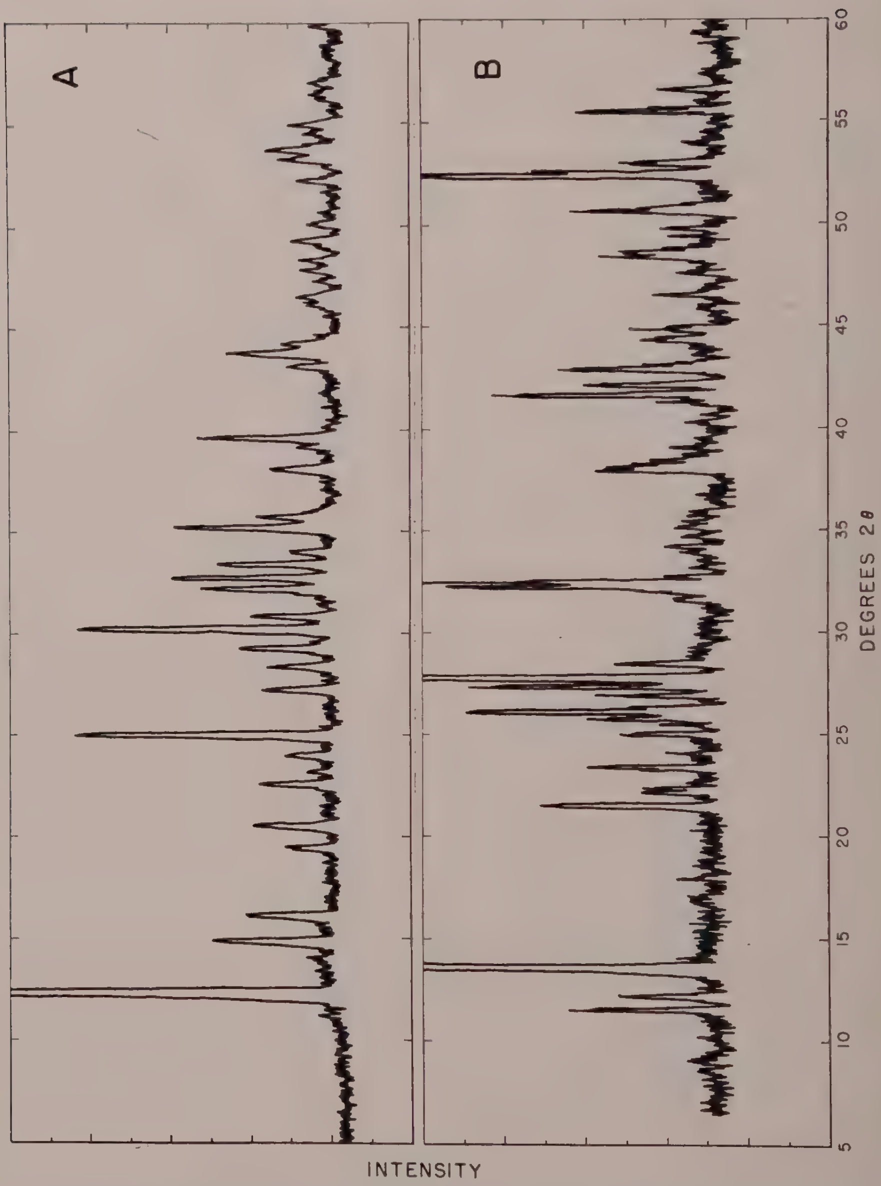

FIGURE 9. X-ray diffraction patterns of crystalline compounds derived from spherulites after prolonged aging in the mother liquor. (A) Compound C: a mixture prepared using 2.04 moles of calcium hydroxide per mole of cupric sulfate (10-6-100 Bordeaux) aged 18 months at room temperature. (B) Compound D: a mixture prepared using 3.38 moles of calcium hydroxide per mole of cupric sulfate (10-10-100 Bordeaux) aged for 24 hours at 45° C. and then for 40 days at 100° C.

TABLE V

COMPOSITIONS OF BORDEAUX CRYSTALLITES, COMPOUNDS C AND D

Constituents	Chemical formulae of compounds	
	C	D
Cu	2.00	1.00
Ca	2.02	1.98
SO_4	0.99	0.98
OH	6.00	4.00
H_2O	3.00	0.90
Apparent density, g./ml.	2.62	2.82

tained from a Bordeaux mixture prepared with 3.38 moles of lime per mole of cupric sulfate by method I. On standing overnight pattern B was obtained, but when the compound was aged in its mother liquor for 25 days at 100° C. it transformed into compound D. When the mixture was put into the steam bath immediately after preparation a minimum of 40 days was required to obtain compound D.

Single crystals of dimensions about $30 \times 10 \times 5\ \mu$ were isolated. Oscillation and Weissenberg diagrams showed the primitive cell to be orthorhombic, but not enough data were available to determine the space group. The cell dimensions are $a = 14.40$ Å, $b = 20.90$ Å, and $c = 7.67$ Å. Calculations from the cell volume, density, and formula weight (Table V) give 12.05 molecules per unit cell. The fact that the layers with h odd are very weak suggests pseudocentering along a.

CONCLUSIONS

In the past freshly prepared Bordeaux mixtures have been considered to be amorphous. X-ray powder patterns show a few Bragg reflections and diffuse bands only 3 minutes after mixing. These patterns were obtained on samples that were washed and dehydrated irreversibly but the structures giving rise to them probably exist in the wet Bordeaux hydrogel since similar reflections were observed on highly concentrated pastes. The intensities were much lower on the patterns of the pastes because of the smaller volume of copper-containing hydrogel due to dilution with water and the presence of gypsum and lime. The Bragg reflections probably arise from small platelets of about 100 Å thickness while the diffuse bands can be indexed as a line-broadened pattern of tribasic cupric sulfate. Evidently part of the material becomes crystalline almost immediately as Feitknecht found in the case of basic cupric nitrate (8). Present evidence does not preclude the presence of an amorphous phase nor is it sufficient to identify the crystalline phases with certainty. The indexing of the diffuse bands as a line-broadened pattern of tribasic cupric sulfate may seem inconsistent since chemical analyses of mixtures having the same age and initial composition show that about 2.5 moles of lime per 4 atoms of copper were bound irreversibly to the hydrogel. A theoretical explanation can be made but can not be supported at present. In any event it is clear that ordered structures appear very soon after mixing and that Bordeaux mixtures are reaction products of polybasic cupric sulfates and calcium hydroxide in various proportions.

The compounds which are obtained depend on the ratio of lime to cupric sulfate and total concentration. Thus at high dilution Fratini and Serra (10) obtained tetrabasic cupric sulfate, $Cu_5SO_4(OH)_8$, and $Cu_5Ca_2SO_4(OH)_{12} \cdot H_2O$ over a range of 1.6 to 3.5 moles of lime. However, in the present investigations where concentrations were based on 10-10-100 Bordeaux, tetrabasic cupric sulfate was not found, while the compound containing 5 atoms of copper to 2 of calcium was formed only when the initial lime content was below about 1.6 moles of lime per mole of cupric sulfate. In many cases the chemical analyses indicated that additional solid phases might be present.

The composition, $Cu_4Ca_4SO_4(OH)_{14} \cdot 3H_2O$, was encountered most frequently within the range of mixtures giving compound B. However, powders containing from 3 to 6 atoms of calcium per 4 atoms of copper could be obtained by varying methods of crystallization and periods of aging. When differences in composition are due to defect structures, then corresponding changes in the relative intensities of diffraction peaks are observed. In this case the only change which occurred can be associated with a different crystalline morphology rather than a higher occupancy of lattice sites or a substitution of one ion for another. Compositions with 3 to 4 calcium atoms per 4 copper atoms gave one pattern and those with 5 to 6 gave a modified one. The pattern associated with the higher calcium content showed improved resolution similar to that obtained with the larger particles of compound A. In all samples, the broader peaks were obtained from spherulites, while the sharper patterns were from extremely small crystallites. Furthermore, these patterns could be indexed provisionally as a hexagonal unit cell with $a = 7.56$ Å and $c = 9.25$ Å containing one formula weight of $Cu_4Ca_4SO_4(OH)_{14} \cdot 3H_2O$. Hence, the differences in compositions probably arise from the presence of at least one additional solid phase. It is assumed that the particle sizes of the other solid phases are very small and consequently contribute only to the background of the diffraction patterns.

On prolonged aging in the mother liquor at room temperature the spherulites (pattern B) prepared with 1.70 to 2.36 moles of lime per mole of cupric sulfate react further to yield $Cu_2Ca_2SO_4(OH)_6 \cdot 3H_2O$. The spherulites prepared with more lime are stable under these conditions, but on heating in the mother liquor at 100° C. they ultimately yield $CuCa_2SO_4(OH)_4 \cdot H_2O$.

Up to the present, 2 polybasic cupric sulfates and 4 polybasic cupric calcium sulfates can be regarded as components of Bordeaux mixtures. The compounds which may occur depend on concentration, ratio of copper to lime, method of preparation and age. It seems probable that further investigations could lead to the preparation of additional compounds in this series, with a more complete elucidation of the structure and composition of Bordeaux hydrogel.

The mechanism of adherence of Bordeaux mixtures to leaf surfaces in terms of the information gained herein is of special interest in their application as fungicides. The tenacity of 10-3.2-100 mixture on foliage under rainfall is great (3). Its property of remaining in suspension, even after long periods of aging, is similar to that of the freshly prepared hydrogel despite the fact that it crystallizes rapidly after mixing. Because the crystallites are very small, of the order of 100 Å, and water is adsorbed, the material remains in suspension. The adherence to foliage of 10-4-100 Bordeaux is comparable to that of the hydrogel, but not quite as good. In this case, again, crystallization into compound A occurs rapidly, although the absorbance at 650 mμ as measured by chelation procedures (5) remains high for a long period of time. This is interpreted to mean that the crystallites are very small and consequently their solubility is relatively high. However, the small crystallite size probably explains its tenacity. Even when it is dried, and the powder rewet, using a small quantity of dispersing agent, its adherence is far superior to that of the spherulites of compound B.

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Note

EFFECT OF VOLTAGE VARIATION ON THE PHOTOMETRIC TITRATION OF FLUORIDES WITH THORIUM NITRATE

R. MAVRODINEANU AND J. GWIRTSMAN

In a previous publication (1) a photoelectric filter photometer was described for the titration of fluorides (F) with thorium nitrate [$Th(NO_3)_4$]. The apparatus is of the balanced type and makes use of two photocells connected in opposition to a sensitive galvanometer. The light bulb of this photometer is connected directly to the 115-v. electric line without any voltage stabilizer. This instrument has been used in the laboratory for over two years with satisfactory results without a voltage stabilizer (1). The voltage fluctuation (114 to 120 v.) seldom exceeds 5 per cent. Other laboratories,[1] however, obtained better results with this photometer when a voltage stabilizer was used.

TABLE I

MILLILITERS OF 0.001N THORIUM NITRATE SOLUTION USED IN THE TITRATION OF KNOWN AMOUNTS OF FLUORIDE ION AT VARIOUS VOLTAGES (SINGLE DETERMINATIONS)

μg. F added	Voltage				
	90	100	110	120	130
0	0.26	0.18	0.14	0.12	0.12
20	1.30	1.14	1.19	1.05	1.01
50	3.05	3.17	3.01	2.85	2.53
80	4.84	4.70	4.58	4.40	3.95

In order to determine the effect of voltage variation, a series of tests was carried out with known amounts of F titrated with $Th(NO_3)_4$ at five different voltages (90, 100, 110, 120 and 130 v.). The variation in voltage during the determinations did not exceed more than 1 per cent. A variation of 10 per cent in voltage resulted in the following percentage decrease in the titration with 0.001N $Th(NO_3)_4$: 2 per cent for 90 to 100 v.; 3.6 per cent for 100 to 110 v.; 4 per cent for 110 to 120 v.; and 6 per cent for 120 to 130 v. (Table I). Corresponding results with 0.01N $Th(NO_3)_4$ were: 6 per cent for 90 to 100 v.; 11 per cent for 100 to 110 v.; 13 per cent for 110 to 120 v.; and 5 per cent for 120 to 130 v. (Table II). The 0.01N $Th(NO_3)_4$ is generally used for titrating solutions containing more than

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[1] Personal communications from Mr. Harold Zeh of Reynolds Metals Company, and Mr. P. A. Puxley of Aluminium Laboratories Limited.

TABLE II

MILLILITERS OF $0.01N$ THORIUM NITRATE SOLUTION USED IN THE TITRATION OF KNOWN AMOUNTS OF FLUORIDE ION AT VARIOUS VOLTAGES
(SINGLE DETERMINATIONS)

μg. F added	Voltage				
	90	100	110	120	130
0	0.11	0.08	0.04	0.03	0.03
200	1.43	1.39	1.31	1.18	1.18
500	3.42	3.27	2.99	2.70	2.61
800	5.10	4.83	4.47	4.01	3.83

200 μg. F. Titrations were carried out using a voltage stabilizer and the results obtained showed no improvement when compared with the titrations carried out without the voltage stabilizer.

When the photoelectric titrator is used with a $0.001N$ $Th(NO_3)_4$ solution and the voltage of the line varies within 5 per cent, the instrument can be used without a stabilizer. The voltage in this laboratory generally varied between 114 and 120 v. resulting in differences in titrations of no more than 1.7 per cent. However, if the voltage varies over 5 per cent, a voltage stabilizer is recommended, especially if the $0.01N$ $Th(NO_3)_4$ solution is used. A voltage stabilizer of the Raytheon type Cat. VR 6112 having a capacity of 60 w. was satisfactory.

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Note

NONINTERFERENCE BETWEEN EFFECTS OF GIBBERELLIC ACID AND FUNGICIDES[1]

LELA V. BARTON AND JEAN MACNAB FINE

Gibberellic acid, a metabolic product of the fungus *Gibberella fujikuroi* (Saw.) Wr. [conidial stage, *Fusarium moniliforme* (Sheld.)], has been the subject of numerous investigations within the last decade. Some authors have shown that bacteria and fungi are not affected by gibberellins, while others have suggested that these substances are antagonistic to several fungi (5). Stowe and Yamaki state (5, p. 210) that "no convincing action of the gibberellins has yet been shown on any organisms except higher plants."

The present paper reports the results of some preliminary greenhouse tests to determine whether it would be feasible to spray for disease control and growth promotion in one operation. Limited slide-germination tests were also made.

GENERAL PROCEDURE

The tomato plant, *Lycopersicon esculentum* Mill., variety Bonny Best, was chosen for study in its response to the disease of early blight [*Alternaria solani* (Ell. & Mart.)]. The bean plant, *Phaseolus vulgaris* L., variety Tendergreen, was used for studies of halo blight [*Pseudomonas phaseolicola* (Burk.) Dowson], and the variety Pinto served as host for the rust [*Uromyces phaseoli* (Pers.) Wint.].

The general procedure for spraying plants was that described by McCallan and Wellman (4), but using a new facility with incubation chambers which accommodate trucks filled with plants. One hundred ml. of test solution were applied with a paint spray gun at 40 lb. air pressure to four replicate pots on a turntable. After spraying and drying, the plants were again placed on the turntable and inoculated with a known suspension of spores, applied with a hand atomizer at 20 lb. pressure for 30 seconds. Immediately after inoculating, the plants were placed on a truck and rolled into an incubation chamber with 95 per cent relative humidity. After 24 hours in the chamber, the plants were returned to the greenhouse.

Tomato plants were grown from seed and were transplanted individually into four-inch pots and allowed to grow for about one month before treatment. Plants selected for uniformity of size were used for each treatment. Bean plants were grown from seeds planted in four-inch pots and thinned to four plants per pot immediately before they were sprayed, about 12 days later. Thus a total of 16 bean plants was used for each treatment since four replicate pots were used.

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[1] This experiment was sponsored by Eli Lilly and Company, Indianapolis, Ind.

Five ml. of 0.5 per cent Triton X-155 were added to each 200 ml. of test solution.

EARLY BLIGHT OF TOMATO

Captan (N-trichloromethyl mercapto-4-cyclohexene-1,2-dicarboximide) in concentrations of 1 and 80 p.p.m. and maneb (manganese ethylene bis-dithiocarbamate) at 3.2, 16.0 and 80.0 p.p.m. were used for spraying the tomato plants. Each captan concentration was used alone and in combination with 1, 10, 50, 100 and 200 p.p.m. gibberellic acid (GA). Maneb concentrations were used alone and in combination with 10 and 100 p.p.m. GA.

A suspension containing 200,000 spores per ml. was prepared immediately before inoculation from cultures of early blight fungus. Infection counts on the third, fourth and fifth compound leaves were made two days after spraying and inoculation. Tests were made on six different dates.

It was shown that GA can be used in combination with either captan or maneb for the control of this disease without decreasing the effectiveness of the fungicides. Also, the presence of the fungicides in the spray mixtures did not reduce the growth-promoting effect of GA on the tomato plant. Furthermore, GA in concentrations of 1, 10, 50, 100 or 200 p.p.m. gave partial control of early blight of tomatoes when used alone or with Triton X-155. The amount of this control varied from test to test (18 to 70 per cent) and all concentrations were equally effective. The disease control noted may be associated with altered physiology of the host plants as evidenced by their extended growth. However, Davis and Halmos (2) reported that the susceptibility of tomato to early blight was not changed by spraying with 20 p.p.m. of potassium gibberellate.

HALO BLIGHT OF BEANS

Agrimycin at 50, 100 and 200 p.p.m. (of active antibiotics) with and without GA at 100 p.p.m. and Agrimycin at 10 and 100 p.p.m. with and without GA at 1, 10, and 100 p.p.m. were used in these tests. Three hours after spraying with these materials, the two primary leaves of each bean plant were injured with a spiked aluminum plate to form 100 infection sites on each leaf, then inoculated with the bacterial culture. After spraying and inoculation, the plants were placed in a greenhouse and the soil watered thoroughly, care being taken not to wet the leaves. Infection counts were made 7 days later on 50 infection sites in each of the two leaves for each plant.

Agrimycin at 0, 50, 100 and 200 p.p.m. gave 0, 92, 100 and 100 per cent control, respectively. When GA at 100 p.p.m. was added to these solutions, the percentages of control were 0, 86, 100 and 100. Thus GA applied alone or in combination with Agrimycin has no effect on the control of halo blight. Growth promotion of the bean plants by GA was significant and was not reduced by Agrimycin.

BEAN RUST

In this case, the bean plants were inoculated two days before spraying with the fungicide. One hundred fifty mg. of fresh rust spores were mixed thoroughly with 1.6 g. of talc and 50 mg. of this mixture were suspended in 200 ml. of distilled water for the inoculum. After inoculation, the plants were placed in a humidity chamber at 60° F. for 24 hours and then taken to the greenhouse. After 24 hours in the greenhouse, they were sprayed with an experimental chemical of unknown composition (known to control the disease) at 80 and 400 p.p.m. with and without GA at 10 and 100 p.p.m. Infection counts were made 7 days after inoculation. The fungicide gave complete control of the disease, which was not affected by GA. Davis and Halmos (2) reported variable effects on the susceptibility of beans to rust infection following spraying with 20 p.p.m. of potassium gibberellate.

SLIDE-GERMINATION TEST

This test (1) was made to determine the effect of GA on the germination of spores of *Alternaria oleracea* Milbraith and *Monilinia fructicola* (Wint.) Honey. A suspension of spores of each fungus was prepared to give approximately 300,000 spores per ml. *Alternaria* spores were diluted by half using orange juice (diluted 100 times with water) as a nutrient and *Monilinia* spores were diluted by half with Lin solution (3). Two ml. each of GA at concentrations of 0, 1, 10, 50, 100, 200, 1000 and 2000 p.p.m. were placed in duplicate test tubes, one for each fungus. To each one of the solutions, 0.5 ml. of each spore-nutrient mixture was added. Two drops of each mixture were then placed on a microscope slide supported by U-shaped glass rods in a Petri dish sealed with water, where the slides were allowed to remain overnight. The number of germinations for 2 × 50 spores of each species for each solution was then counted.

Averages of duplicate counts for *Alternaria* showed 86 to 95 per cent germination without GA or with GA at 1, 10, 50, 100 and 200 p.p.m. There was no germination of these spores in GA at 1000 or 2000 p.p.m. Spores of *Monilinia*, on the other hand, gave 85 to 95 per cent germination at all concentrations of GA, including 1000 and 2000 p.p.m. The original test tubes containing all of these cultures were kept another day and the spores reexamined. It was found that the *Alternaria* still had not germinated at the two highest concentrations of GA and that *Monilinia* continued to grow as well at 1000 and 2000 p.p.m. GA as at the lower concentrations.

Thus a toxic dosage does not seem to have been approached in the latter case, while the *Alternaria* germination was affected by high concentrations of GA. This sensitivity of *Alternaria*, though of a different species, may be related to the partial control of early blight of tomato by GA.

CONCLUSIONS

It is concluded that GA probably can be used with fungicides and bactericides without any interference with the control of diseases and, further, that the commercial compounds do not impair the growth-promoting properties of the GA. Also, the differential response of *Alternaria* and *Monilinia* spores to GA and the partial control of early blight of tomato by GA point to the possibility of the control of some fungi by GA itself.

In cases where the application of GA would be advantageous, it would be feasible to spray for disease control and growth promotion in one operation.

ACKNOWLEDGEMENT

The authors wish to express their thanks to Mr. Alvaro Goenaga for fungus cultures and for valuable assistance in the execution of these experiments.

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APPLICATION OF CHOLINESTERASE ASSAY TO RESIDUE ANALYSIS OF 1-NAPHTHYL *N*-METHYLCARBAMATE (SEVIN[1])[2]

Herbert H. Moorefield and Edwin R. Tefft

SUMMARY

The cholinesterase technique, now in use for estimating residues of various organic phosphate pesticides, has been satisfactorily adapted for use with carbamate insecticides such as Sevin (1-naphthyl *N*-methylcarbamate). Excellent recovery of fortified samples from diversified plant products such as lettuce, peas, pea pods, apples, and foliage of beans and cotton has been demonstrated. Results of the enzymatic determination of residues on field-treated apples has been verified by chemical analysis. In the general procedure, the commodity is stripped with chloroform, the solvent evaporated, and the Sevin is estimated enzymatically by quantitating its anticholinesterase activity. The method as described is sensitive to 0.5 microgram of Sevin.

INTRODUCTION

With the advent of Sevin into the pesticide field (5), the potential development of a novel class of general, broad-activity, agricultural insecticides has been opened. The carbamates can be quantitatively estimated for the purpose of residue analysis by means of the 4-aminoantipyrine reaction (3, 6). However, unless special precautions are taken, natural phenolic plant components and degradative hydrolysis products of the carbamates themselves can possibly interfere and bias the results.

The ability of the organic phosphate insecticides to inhibit cholinesterase has been used as the basis of an enzymatic method of analysis (2) to estimate residues of several different phosphorus compounds (9) and official recognition of this technique has been given by the Food and Drug Administration (10). Because Sevin is also an anticholinesterase (5), the present study was undertaken to demonstrate that enzymatic determination of residues can be successfully adapted to cover the carbamate insecticides.

MATERIALS AND METHODS

Preparation of enzymes. Mass collections of house fly (*Musca domestica* L.) heads were made by a previously described method (8). These were thoroughly macerated with a mortar and pestle in pH 7.9 buffer ($0.15M$ NaCl, $0.04M$ $MgCl_2$, and $0.025M$ $NaHCO_3$). After establishing the titer of cholinesterase activity in this tissue by means of manometric assay, the concentrated brei was distributed into test tubes, corked and stored in a deep freeze.

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[1] Sevin is the registered trademark of Union Carbide Corporation.

[2] This investigation was sponsored by a Union Carbide Chemicals Co. Fellowship. This article was preprinted May 15, 1958.

The mammalian enzymes were obtained from recently outdated blood furnished by the American Red Cross. Plasma, containing the serum esterase, was separated from centrifuged blood, diluted with bicarbonate buffer, assayed and frozen in tubes. The packed erythrocytes were washed three times with 0.9 per cent NaCl, haemolyzed in dilute HCl, diluted in bicarbonate buffer, assayed and also dispensed into tubes prior to freezing.

Equivalent enzyme preparations of the three esterases were derived by predetermining the volume of brei delivered into each tube so that when the contents were thawed and diluted to 100 ml., a standard enzyme solution resulted that would release approximately 300 microliters of CO_2 from the bicarbonate buffer in 30 minutes, under the prescribed conditions. These frozen preparations remained stable for at least six months without loss of cholinesterase activity.

Sample processing. All of the samples treated (except apples) were weighed and homogenized with chloroform in a Waring Blendor. Fortification was made by addition of precise amounts of purified Sevin, dissolved in chloroform, into the pulp as described by Gunther and Blinn (4, p. 13). The stripping solution was then decanted through a double thickness of cheesecloth and filtered. Quantitative recovery was obtained by successive rinses of the mince with small volumes of solvent. Removal of the chloroform was accomplished by immersion of the flask containing the stripping solution in a warm water bath and impinging a gentle stream of filtered air onto the surface of the liquid. Last traces of the solvent were removed at room temperature. The residue was dissolved in acetone and diluted to a minimum known volume.

The apple pulp formed stable emulsions when blended with chloroform; acetone was substituted as the stripping solvent. After reduction of volume of the filtrate, water was removed with anhydrous Na_2SO_4. The insecticide was recovered from the residue with fresh acetone and the procedure followed as outlined above.

Untreated control samples of each crop were handled identically as those fortified in order to evaluate the possible presence of endogenous interfering substances.

Cholinesterase assay. These determinations were made manometrically by the technique described by Metcalf and March (7). Two and six-tenths ml. of the enzyme solution in bicarbonate buffer were delivered into the main compartment of each Warburg flask, and 0.3 ml. of $0.2M$ acetylcholine bromide into the side arm of the vessel. One-tenth ml. of the standard solution of Sevin or plant extract was added to the brei bringing the total volume to 3 ml. A mixture of 95 per cent N_2 and 5 per cent CO_2

was flushed through the flasks for 5 minutes and, after an additional 10-minute equilibration period, the substrate was tipped into the enzyme solution. Readings of the released CO_2 were made following a 30-minute period of agitating the reaction mixture at a bath temperature of 37° C.

Percentage inhibition was determined by comparing the average microliters of gas released in the paired, inhibited test vessels at a given concentration of Sevin, with the mean gas volume produced in duplicated, uninhibited controls. Standard curves were derived from paired assays consisting of six different concentrations of Sevin. The determined values of Sevin residue are averages of two analyses, each made in duplicate in separate Warburg assays.

Chemical determination. Sevin was extracted with chloroform in a Soxhlet extractor from finely shredded apple samples. In the confirmatory tests aliquots of these solutions were assayed by the enzyme technique and the remainder washed with dilute HCl to remove water-soluble impurities. The volume of each was concentrated in a Kuderna-Danish evaporator, the fruit waxes precipitated and the 1-naphthyl *N*-methylcarbamate hydrolyzed to 1-naphthol with NaOH. Analyses were completed spectrophotometrically by means of the 4-aminoantipyrine reaction. This method of residue determination has been described in detail (1).

RESULTS

The comparative antiesterase assays, given in Table I, show the relative effectiveness of Sevin as an inhibitor of equivalent preparations of enzymes from house fly brain, human blood plasma and erythrocytes, when measured under standardized conditions. The results indicate that Sevin shows a 10-fold greater affinity for the insect-derived, house fly cholinesterase than for human red blood cell cholinesterase. Inhibition of the blood serum or pseudoesterase by Sevin is even less efficient as demonstrated by the I_{50} values (molar concentration required to inactivate one-half of the enzyme activity).

The calibration curve used in this study, plotting p.p.m. of Sevin against inhibition of the house fly cholinesterase, is graphically depicted in Figure 1. Values can be directly interpreted as micrograms of carbamate per ml. of stripping solution. This curve is the average result of three in-

TABLE I

COMPARATIVE AFFINITY OF SEVIN FOR VARIOUS ESTERASES

Enzyme source	I_{50} (Molar concentration of Sevin required to effect 50% cholinesterase inhibition)
House fly brain	3.4×10^{-7}
Human red blood cell	3.0×10^{-6}
Human blood serum	6.4×10^{-6}

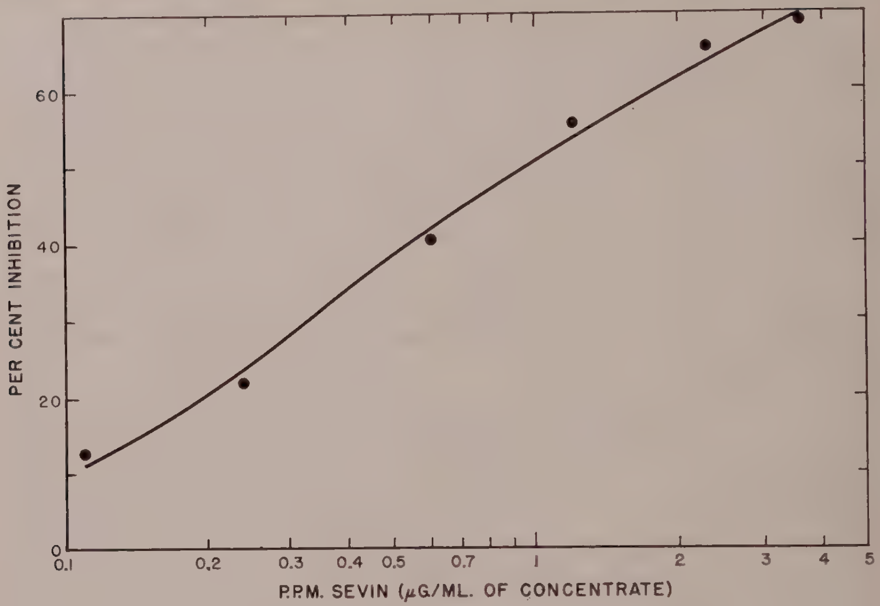

FIGURE 1. Inhibition of house fly cholinesterase by Sevin.

dependent, duplicated assays, each made with freshly prepared carbamate solutions. The line was derived by a cubic equation by the method of least squares. Expressed in terms of concentration of Sevin, the range covered in this plot is 6×10^{-7} to 2×10^{-8} moles.

Different representative types of plant tissue were selected to test the efficiency of the enzyme assay for analysis of Sevin residues (Table II). A wide span of insecticide concentrations, from 0.01 to 10.0 p.p.m., was used for fortification to assess limits of sensitivity of the method. No serious interference from endogenous inhibitors of cholinesterase was encountered in untreated extracts of the crops tested. Statistical treatment of the data on the fortified samples of various plant products is shown in Table III. In order to equally weight the recovery figures, where determinations were performed over such a wide concentration range, the formula, $b = \Sigma xy/\Sigma x^2$, was used to calculate the average per cent recovery on each type of sample ($x = \mu$g. Sevin added, $y = \mu$g. Sevin recovered) (11, p. 47). Confidence limits at the 95 per cent level, based on the data presented, have also been deduced for each crop.

To verify the results obtained by the anticholinesterase residue method, confirmatory tests were performed by chemical analysis. Three samples of field-treated apples were harvested and extracted with chloroform. The Sevin contained in this stripping solution was measured by both chemical and enzyme assays. Results are presented in Table IV. A fourth batch of

TABLE II

CHOLINESTERASE DETERMINATION OF SEVIN IN FORTIFIED SAMPLES

Sample	Sample Wt. (g.)	Sevin added		Sevin recovered	
		p.p.m.	µg.	µg.	%
Lettuce (*Lactuca sativa* L.)	12	0.1	1.2	1.3	108
	12	0.5	6.0	6.3	105
	12	1.0	12.0	11.4	95
	12	5.0	60.0	63.0	105
	12	10.0	120.0	117.0	98
Peas (*Pisum sativum* L.)	12	0.1	1.2	1.1	92
	12	0.5	6.0	5.9	98
	12	1.0	12.0	11.1	93
	12	5.0	60.0	66.0	110
	12	10.0	120.0	115.9	97
Pea pods (*P. sativum* L.)	12	0.1	1.2	1.0	83
	12	0.5	6.0	6.0	100
	12	1.0	12.0	12.6	105
	12	5.0	60.0	60.0	100
	12	10.0	120.0	123.0	103
Bean foliage (*Phaseolus vulgaris* L.)	12	0.1	1.2	1.1	92
	12	0.5	6.0	6.0	100
	12	1.0	12.0	13.8	115
	12	5.0	60.0	61.5	103
	12	10.0	120.0	114.0	95
Cotton foliage (*Gossypium hirsutum* L.)	12	0.1	1.2	1.1	88
	12	0.5	6.0	5.8	96
	12	1.0	12.0	12.4	103
	12	5.0	60.0	66.0	110
	12	10.0	120.0	108.0	90
Apple pulp (*Malus sylvestris* Mill.)	800	0.01	8.0	9.5	118
	400	0.1	40.0	40.0	100
	400	0.5	200.0	199.0	100
	400	1.0	400.0	385.0	96

TABLE III

STATISTICAL ANALYSIS OF RECOVERY DATA ON SAMPLES FORTIFIED WITH SEVIN

Sample	Average % of added Sevin recovered	95% Confidence limits
Lettuce	99.0	94.0–104.0
Peas	99.2	94.2–104.2
Pea pods	102.0	97.0–107.0
Bean foliage	96.6	91.6–101.6
Cotton foliage	94.1	89.1– 99.1
Apple pulp	96.9	92.0–101.9

TABLE IV

COMPARATIVE RESULTS OF CHOLINESTERASE AND CHEMICAL ANALYSES OF SEVIN RESIDUE ON FIELD-TREATED APPLES

Sample		p.p.m. Sevin	
Weight (g.)	Stage of preparation analyzed	Cholinesterase determination	Chemical determination
200	Stripping solution	5.2	5.6
200	Stripping solution	2.1	2.1
200	Stripping solution	5.4	4.8
400	Whole apples	4.0	4.3

apples was divided into two lots and independent analyses were made by each method, starting with whole fruit. Excellent agreement of results from the two analytical techniques was obtained.

DISCUSSION

The experimental results indicate that the anticholinesterase assay should provide an accurate means of determining residues of the carbamate insecticides in a wide variety of commodities. Sensitivity of the method is dependent on the effectiveness of Sevin as a cholinesterase inhibitor. Although the fly brain was used as an enzyme source in these studies, Sevin is sufficiently active against red blood cell cholinesterase that commercial preparations could be substituted. The variance in affinity of Sevin for insect versus mammalian cholinesterase suggests a possible role in the differential toxicity previously reported (5).

No interference has been encountered in the cholinesterase assay from insecticides such as: DDT, BHC (technical benzene hexachloride, gamma isomer 20 per cent), chlordane, toxaphene, dieldrin, aldrin, rotenone, or pyrethrins (20 per cent extract) (2). As the phosphorus insecticides are also strong anticholinesterases, mixed residues containing Sevin and any of these compounds could not be determined by this enzyme assay unless efficient analytical techniques were developed to separate these materials in processing of the samples.

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DETERMINATION OF INDIVIDUAL FUNGUS SPORE VOLUMES AND THEIR SIZE DISTRIBUTION[1]

S. E. A. McCallan

SUMMARY

Spore volumes were obtained from linear measurement by assuming an appropriate geometric shape such as a prolate spheroid. Measurements were made of 50 to 400 spores from each of 24 species of fungi. The mean spore volumes in cubic microns were as follows: *Uromyces caryophyllinus* 15,600, *Alternaria solani* 9960, *Stemphylium sarcinaeforme* 8680, *Erysiphe polygoni* 7550, *Puccinia antirrhini* 6180, *Uromyces phaseoli* 6110, *Phytophthora infestans* 5360, *Tilletia caries* 3730, *Venturia inaequalis* 616, *Botrytis cinerea* 556, *Venturia pyrina* 546, *Monilinia fructicola* 523, *Alternaria oleracea* 497, *Rhizopus nigricans* 323, *Pestalotia stellata* 321, *Glomerella cingulata* 292, *Neurospora sitophila* 283, *Ustilago maydis* 259, *Neurospora crassa* 99.3, *Aspergillus niger* 29.2, *Myrothecium verrucaria* 26.8, *Penicillium expansum* 16.6, *Phyllosticta* sp. 11.2 and *Cephalosporium acremonium* 5.06. Coefficients of variation ranged from 14 to 100 per cent.

The size distribution was determined by χ^2 and t tests for symmetry and kurtosis. The distribution of 7 species was normal, of 12 log-normal, of 2 intermediate, and for 3 (due to limited data) both types of distribution were indicated. Of the species with smaller mean spores, i.e., less than 500 μ, 3 tend to log-normal distribution, and species of the same genus tend to the same kind of distribution.

The stability of mean spore volume appears constant for some species, but varies significantly for others depending on the particular lot collected and time maintained in culture. Different mechanical methods of collecting conidia of *M. fructicola* had no effect on spore volume.

Comparisons of spore weights derived from volume measurements (density 1.1) with direct weighings for six species gave good agreement for two but were somewhat higher for four species.

INTRODUCTION

It has long been customary in the identification of fungus species to include a simple statement of the range of spore lengths and widths. The next step, more rarely taken, has been a calculation of the mean length and width together with the standard deviation. More limited information is available on the lengths and widths of spores of a given species and the ratio of one to another, as for example the extensive measurements of Levine (6) in an attempt to show morphological differences between the uredospores of various biological strains of *Puccinia graminis tritici*, or those of Stevens (19) on the saltants of *Helminthosporium sativum*. Van

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[1] This work was supported in part by a grant from the Rockefeller Foundation. A preliminary report was presented before The American Phytopathological Society, Palo Alto, California, August, 1957 (8).

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Luijk (21) has used frequency curves of spore lengths and widths as an aid in delimiting genera. However, beyond this type of comparison there appears to be relatively little information on spore dimensions.

In the course of various studies on the nature of fungicidal action reported since 1930 (9, 10, 11, 15, 16), measurements have been made on spore volume and also on density with the object of obtaining the fresh weight of a given number of spores. Such weights are necessary for determining the fungicide dose and other reactions involving the spores where the responses are to be measured on a fresh weight basis and the amount of spores is limited.

The purpose of the present communication is to report on an examination of some of these data together with various new measurements in order to determine the nature of the distribution of spore sizes based on volume. Some data are also given on the stability of the volume sizes. Volume measurements are given on the spores of 24 species of fungi including many of those commonly used in fungicide studies. It is hoped that these values also may be of use to other workers.

MATERIAL AND METHODS

Spores of 24 different species have been obtained and with the exception of the smut spores were all in the conidial form. The species and references to source of spores and previously reported measurements from this laboratory are as follows:

Alternaria oleracea Milbraith (7, 10, 15)
Alternaria solani (Ell. & Mart.) Jones & Grout
Aspergillus niger van Tiegh (9, 15)
Botrytis sp. (*cinerea* type) (11, 22)
Cephalosporium acremonium Corda (15)
Erysiphe polygoni DC.
Glomerella cingulata (St.) Sp. & von S. (10, 11, 15)
Monilinia fructicola[2] (Wint.) Honey (15)
Myrothecium verrucaria (Alb. & Schw.) Ditm. ex Fr. (9)
Neurospora crassa Shear & Dodge
Neurospora sitophila (Mont.) Shear & Dodge (12, 15)
Penicillium expansum Link[3]
Pestalotia stellata B & C. (11)
Phyllosticta sp.[4]

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[2] Reported earlier as *Sclerotinia americana* (Wormold) Norton & Ezekiel (11, 22), and *S. fructicola* (Wint.) Rehm. (10, 13).

[3] Culture furnished by Dr. G. B. Ramsey, United States Department of Agriculture, 5630 Ingleside Ave., Chicago, Ill.

[4] Chosen as representative of pycnidiospores. Isolated by Mr. A. Goenaga of this Institute from a polyploid of *Verbena hybrida* in the greenhouse and does not appear to agree with either of the two species reported from *Verbena* by Seaver (17).

Phytophthora infestans (Mont.) deBary
Puccinia antirrhini Diet. & Holw. (11)
Rhizopus nigricans Ehr. (9, 10, 15)
Stemphylium sarcinaeforme (Cav.) Wilts. (7, 10, 11, 15, 22)
Tilletia caries (DC.) Tul. Race T-14[5]
Uromyces caryophyllinus (Schr.) Wint. (11, 22)
Uromyces phaseoli (Pers.) Wint.
Ustilago maydis (DC.) Cda.
Venturia inaequalis (Cke.) Wint. (11, 16)
Venturia pyrina Aderh. (16)

The spores of the majority of the species were obtained from cultures grown on potato dextrose agar. Exceptions were the species of *Neurospora* which were grown on Horowitz medium (5) with the omission of DL-methionine, and the sporangia of *P. infestans* which were obtained from cultures on wheat kernels. The conidia of *V. inaequalis* and *V. pyrina* were collected from naturally infected fruits of apple (*Malus sylvestris* Mill.) and pear (*Pyrus communis* L.). Likewise the uredospores of *P. antirrhini*, *U. caryophyllinus* and *U. phaseoli* were collected from infected plants in the greenhouse, respectively, of snapdragon (*Antirrhinum majus* L.), carnation (*Dianthus caryophyllus* L.), and bean (*Phaseolus vulgaris* L. var. Bountiful), and the conidia of *E. polygoni* from the Pinto variety of bean. Spores of *T. caries* were obtained from infected wheat kernels (*Triticum aestivum* [L.]) and of *U. maydis* from naturally infected local plantings of sweet corn (*Zea mays* L. var. *saccharata* [Sturtev.] Bailey).

The spores were washed from the medium, excepting *Neurospora* spores which were obtained by the vacuum technique (12), washed and centrifuged and resuspended in water. Mounts were examined under oil immersion at a magnification of 1455× (except for the uredospores, and conidia of *A. solani*, *E. polygoni*, and *S. sarcinaeforme* which were examined under high power), and the length and width of individual spores determined with an ocular micrometer. Spores of many species were measured to the nearest half ocular space, i.e., 0.615 μ, and several to the nearest one-quarter space. Six species examined earlier (11) were measured to the nearest micron.

The shape of the spores of all but two of the species appeared to be adequately defined as a prolate sphaeroid and accordingly the volume of each spore was so calculated from the simplified formula, $0.5236 \times \text{length} \times \text{width}^2$. Spores of *Alternaria oleracea* were considered to be defined as a frustrum of a cone with hemispherical ends; this necessitated three basic measurements, while those of *A. solani* were described as a frustrum of a cone with a hemispherical cap on the large end and a cylindrical beak on the smaller end; this required four basic measurements. The data that follow were based on measurements of 100 spores with many of the species; however, with the 6 species measured at an early date (11) only 50 spores were examined. With three other species, i.e., *Monilinia fructicola*, *Aspergillus niger* and *Neurospora sitophila*, several lots of 100 spores measured at different times were available for study. In order to eliminate possible bias in selecting the spores for measurement, a mechanical stage was used in all but the early studies, and various devices employed to select the spores at random. For example, as the stage was moved horizontally the first spore to appear from outside the field was brought to the center of the stage and measured, then the next spore outside the field was brought in and measured and so on. In addition several freshly made mounts were examined.

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[5] Smut balls furnished by Dr. C. S. Holton, State College of Washington, Pullman, Wash.

The limitations of the method of measuring the spore lengths and widths necessarily reduced the number of different possible sizes and in effect introduced the error of grouping; however, it is not believed that this detracts from the main conclusions. Measurements on fairly large spores gave a wide range of lengths and widths; for example with *Monilinia fructicola*, 4 lots of 100 gave a total of 16 different lengths and 10 different widths. The former ranged from 5.5 to 14.6 μ and the latter from 4.9 to 11.0 μ. Combinations of these gave 60 different volumes ranging from 69 to 924 cubic μ. With the smaller spores the limitations became more marked; thus with *Aspergillus niger* there were obtained only 6 different lengths and 5 widths, resulting in 20 different volumes. Spores of *Cephalosporium acremonium*, *Penicillium expansum*, and *Myrothecium verrucaria*, although smaller, were measured with greater precision and thus gave more classes. All spore volumes were calculated to three significant figures.

When measurements of more than one lot of 100 spores of a given species obtained under different circumstances were available, the different lots were examined by the analysis of variance (18) to determine if they were from a homogenous population before they were combined for further study.

A preliminary inspection of the data indicated that the spore volumes of a number of the species would be normally distributed while others might show a log-normal distribution. This comparison was made graphically by plotting the cumulative percentage of spores against the different sizes on arithmetic- or logarithmic-probability paper. A straight line response would indicate a reasonable normal or log-normal distribution, respectively. Curves concave downwards on an arithmetic plot indicated the possibility that the volumes should be converted to logarithms.

In the large samples departures from normality, as shown by tests for skewness and kurtosis (18) can be shown more readily than in the small samples. It is also more difficult to set up objectively the class limits with smaller samples. Thus it was decided also to apply the χ^2 test to determine the extent of the deviation from the normal distribution.[6] This method does not evaluate skewness or kurtosis alone but gives an over-all measure of departure from normality. The mean and standard deviations of the individual spore volumes or of their logarithms were determined (18, p. 200). From a table of the normal distribution (4, Table I) the factor was obtained for defining the limits for each 10 per cent of the area beneath the normal distribution curve. For example, the mean plus or minus 0.2533 times the standard deviation will give the two 10 per cent limits adjacent to the mean. The expected frequency being known within these limits, it was possible to compare the observed frequencies by means of the χ^2 test. A division into 10 per cent classes gave an expected value of 5 for the samples of 50 which is the minimum recommended (18, p. 30), while for the largest sample a theoretical value of 40 was available.

The samples consisting of 50 or more different spore sizes were grouped into 19 to 21 classes for the customary t test for symmetry and kurtosis (18, p. 200). For intermediate samples fewer classes were used. However, for all samples consisting of less than 30 different sizes, or where it was questionable as to whether the distribution was normal or log-normal, the t test was made on the individual spore volumes, nongrouped, as suggested for small samples (18, p. 199) but in addition values for g_2 were computed.

RESULTS

The basic linear dimensions for the spores of the 24 species are given in Table I. The results of the tests for the type of distribution are shown in Table II. It will be seen in Table II A, that 7 species showed a normal distribution with no pronounced departures from the expected as shown by the χ^2 test and no very marked indications of asymmetry or kurtosis. The 12 species believed to be log-normally distributed are given in Table II B. In most cases the fit is reasonably good; however, there are some exceptions, particularly with *Erysiphe polygoni* which shows marked positive kurtosis and *Neurospora crassa* with a high χ^2 value. Examples of cumulative frequency distributions of spore volume sizes are shown in Figure 1 for several normally or log-normally distributed species. The calculated linear curve for normal distribution has also been drawn and is indicative of the goodness of fit. An excess of the smallest size spores can be noted especially for *Glomerella cingulata* and to a lesser extent for *Monilinia fructicola;* however, this departure is not very marked.

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[6] The author is indebted to Mr. Charles W. Dunnett, Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York, and Dr. Frank Wilcoxon for suggesting this form of applying the χ^2 test.

TABLE I

LINEAR DIMENSIONS OF SPORES IN MICRONS

Species	Length		Width	
	Mean	Range	Mean	Range
Alternaria oleracea	18.4	7.3–39.0	Major 7.8 Minor 3.7	5.0–11.0 2.4–7.3
Alternaria solani	Body 66.2 Beak 31.0	32.9–108.2 11.0–74.0	Major 19.3 Minor 4.3	13.7–27.4 2.7–8.2
Aspergillus niger	4.0	2.3–5.3	3.6	2.3–4.9
Botrytis cinerea†	11.7	6 –15	9.3	6 –12
Cephalosporium acremonium	3.5	2.3–6.5	1.6	0.9–2.1
Erysiphe polygoni	37.9	25.3–48.6	19.50	16.4–24.7
Glomerella cingulata	15.5	7.4–23.5	5.9	4.3–8.7
Monilinia fructicola‡	12.0	5.5–14.6	9.0	4.9–11.0
Myrothecium verrucaria	7.0	5.5–8.3	2.7	2.4–3.1
Neurospora crassa	6.7	3.5–10.6	5.0	3.5–8.2
Neurospora sitophila††	8.8	3.5–20.0	7.4	2.4–12.8
Penicillum expansum	3.3	2.8–4.2	3.0	2.8–3.5
Pestalotia stellata†	20.5	18 –27	5.4	4 –7
Phyllosticta sp.	4.5	3.1–7.4	2.1	1.5–2.9
Phytophthora infestans	14.2	9.3–22.2	8.0	6.2–11.1
Puccinia antirrhini†	24.4	28 –20	21.9	20 –24
Rhizopus nigricans	8.8	4.3–15.3	7.9	4.3–11.6
Stemphylium sarcinaeforme†	28.2	20 –38	23.6	14 –30
Tilletia caries	20.3	17.8–24.0	18.7	16.6–21.5
Uromyces caryophyllinus†	27.3	23 –33	23.8	20 –28
Uromyces phaseoli	25.7	21.2–30.8	21.3	18.5–26.0
Ustilago maydis	8.2	6.8–9.5	7.7	6.1–8.9
Venturia inaequalis†	19.3	14 –24	7.7	4 –10
Venturia pyrina	21.7	12.2–34.2	6.8	4.9–9.8

100 Spores unless otherwise indicated: † = 50 spores, †† = 300 spores, ‡ = 400 spores.

An intermediate group of 5 species is shown in Table II C. With 3 of these, namely *Uromyces caryophyllinus*, *Puccinia antirrhini*, and *Pestalotia stellata*, due to limited data, both normal and log-normal distributions appear reasonable, though for *P. stellata* a log-normal distribution would seem more probable. With *Neurospora sitophila* and *Aspergillus niger* departures from both types of distribution were very marked and these two species are apparently intermediate. This is shown graphically in Figure 2. However, *N. sitophila* is clearly much closer to a log-normal than a normal distribution. An example of a species, *Puccinia antirrhini*, apparently fitting both distributions is also given in Figure 2.

Graphic examples of the bell-shaped distribution curves are illustrated in Figure 3 for a normally distributed species, *Monilinia fructicola*, based on a large number of spores, and for a log-normally distributed one, *Myrothecium verrucaria*, based on more limited data. In Figure 3 the class marks are plotted rather than the more common histograms.

The mean spore volumes and standard deviations are summarized for the 24 species in Table III into the two main types of distribution, with

TABLE II

TESTS FOR DISTRIBUTION OF SPORE VOLUME SIZES

A. Normal distribution

Species	Total spores	Different sizes	χ^2 8 D. F.	*t* Values	
				Symmetry	Kurtosis
Stemphylium sarcinaeforme	50	40	10.4	0.89	0.80
Tilletia caries	100	39	7.2	2.00*	2.31*
Venturia inaequalis	50	25	4.4	0.30	−0.25
Botrytis cinerea	50	24	16.8*	0.26	−0.24
Venturia pyrina	100	40	7.2	0.93	1.39
Monilinia fructicola	400	65	20.0*	−0.78	0.24
Ustilago maydis	100	34	3.8	0.17	−0.16
B. Log-normal distribution					
Alternaria solani	100	98	14.4	−1.50	0.64
Erysiphe polygoni	100	74	25.2**	0.47	2.63**
Uromyces phaseoli	100	56	4.2	1.58	0.60
Phytophthora infestans	100	75	5.8	0.53	−0.24
Rhizopus nigricans	100	38	17.8*	−1.36	0.40
Alternaria oleracea	100	81	17.8*	0.75	0.66
Glomerella cingulata	100	71	5.8	2.05*	2.05*
Neurospora crassa	100	17	36.8**	0.92	−1.07
Myrothecium verrucaria	100	38	18.6*	0.98	−0.66
Penicillium expansum	100	26	7.2	1.11	0.45
Phyllosticta sp.	100	46	17.0*	−0.48	−0.73
Cephalosporium acremonium	100	23	28.6**	1.60	−0.34
C. Normal and log-normal or intermediate distribution					
Uromyces caryophyllinus Normal	50	32	3.6	1.25	2.03*
Uromyces caryophyllinus Log-n.	50	32	7.2	−1.27	1.80
Puccinia antirrhini Normal	50	24	5.6	0.32	1.01
Puccinia antirrhini Log-n.	50	24	4.0	−0.63	−0.77
Pestalotia stellata Normal	50	20	18.0*	1.89	0.85
Pestalotia stellata Log-n.	50	20	16.8*	−0.74	−0.42
Neurospora sitophila Normal	300	38	73.9**	12.15**	9.46**
Neurospora sitophila Log-n.	300	38	48.7**	−5.37**	5.20**
Aspergillus niger Normal	300	22	114.1**	3.22**	0.02
Aspergillus niger Log-n.	300	22	186.7**	−4.56**	1.58

*=Significant at the 5 per cent level.
**=Highly significant at the 1 per cent level.

the intermediate assigned to one or the other. For these 24 species the range in mean volumes is 3000-fold from the largest, *Uromyces caryophyllinus*, to the smallest, *Cephalosporium acremonium*. The smaller-spored forms, i.e., those with means less than 500 μ, including *Aspergillus niger*, are (with one exception *Ustilago maydis*) all log-normally distributed, but the larger-spored species are present in both groups. There is also a tendency for species of the same genus to have the same type of distribution.

The standard deviations for the normally distributed spores, Table

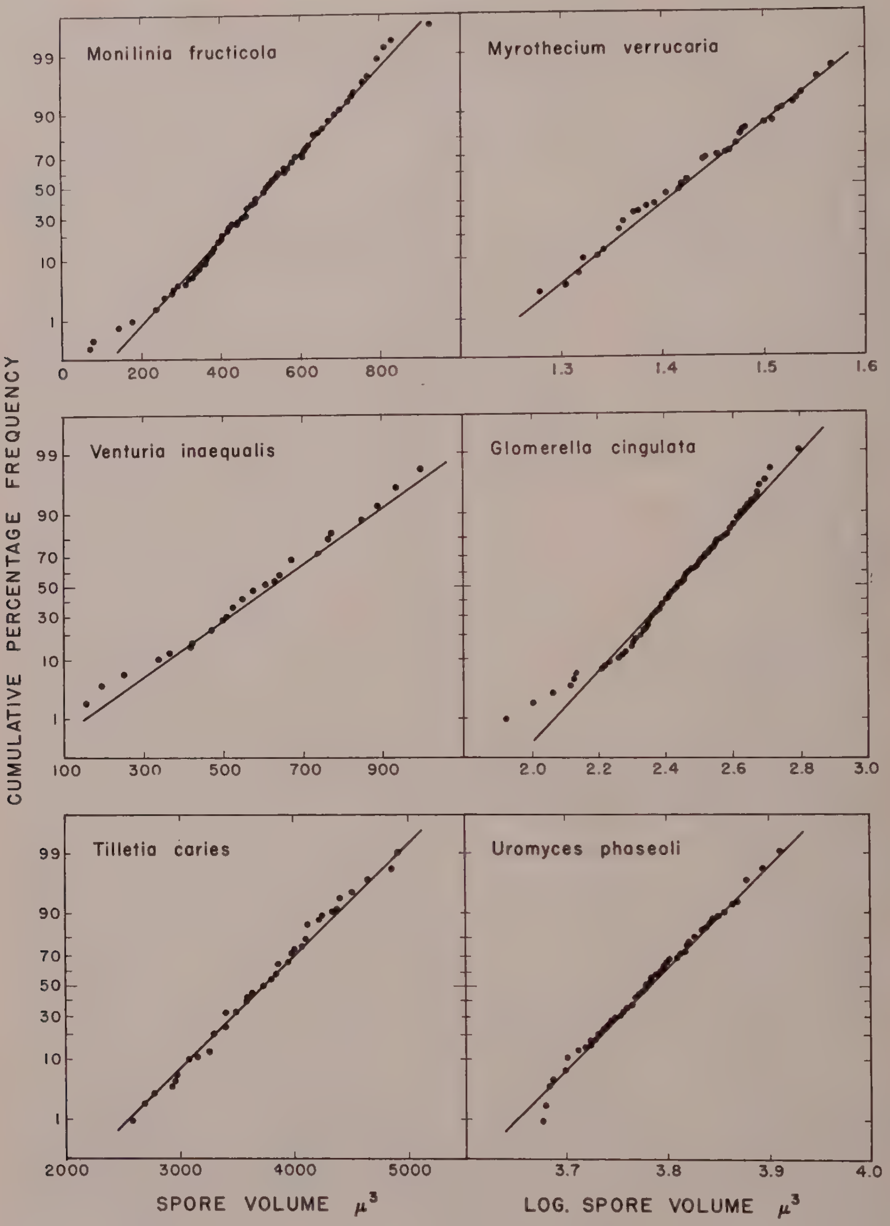

FIGURE 1. Examples of cumulative frequency distributions for spore volumes normally distributed and log-normally distributed.

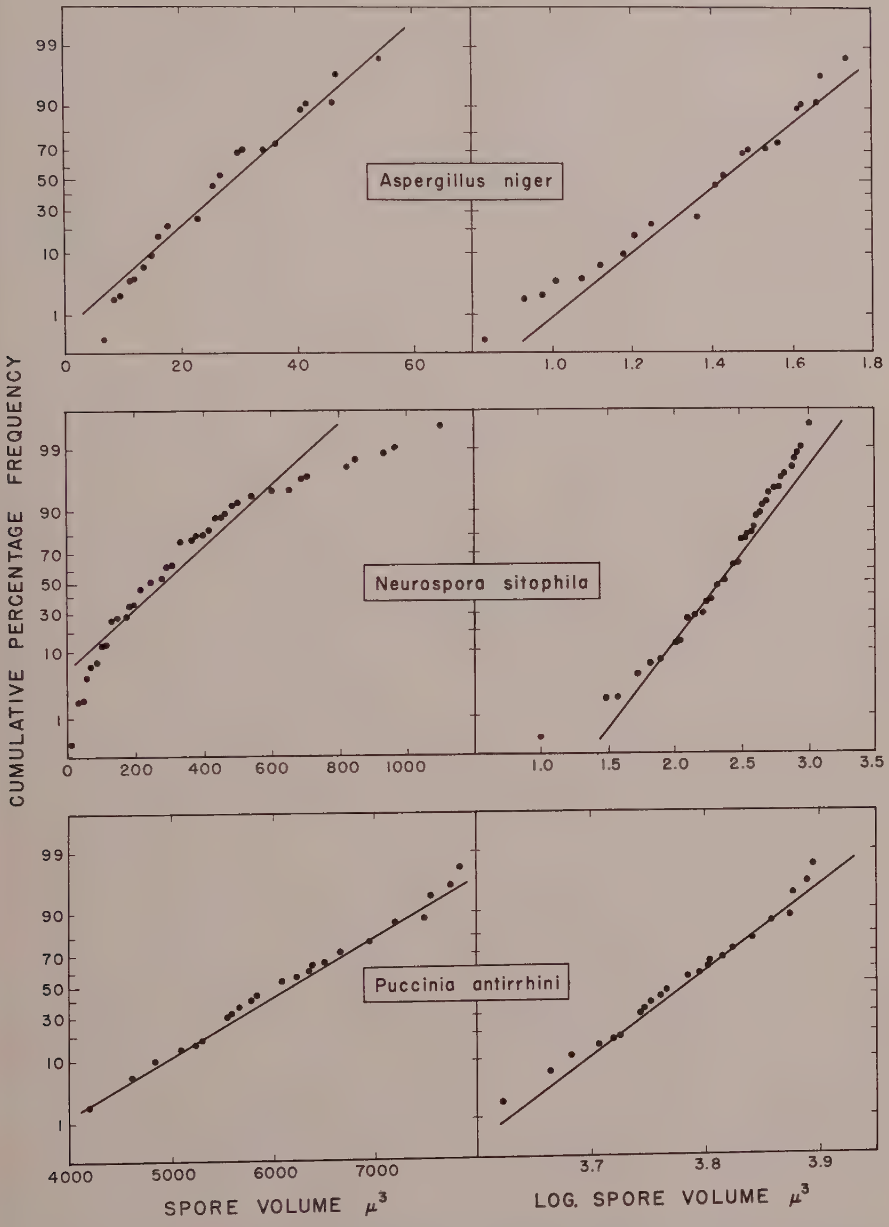

Figure 2. Examples of spore volumes intermediate between a normal and log-normal distribution (*A. niger* and *N. sitophila*) and a species (*P. antirrhini*) following both types of distribution.

III A, expressed as coefficients of variation do not vary widely and range only from 15 to 39 per cent. For the sake of comparison the log-volumes of the log-normally distributed spores have been expressed as antilogarithms and here the standard deviations will appear as ratios. Thus *Alternaria solani* will have a coefficient of 45 per cent. It will be seen that the range in coefficients of variation is much larger for this second group, ranging from 14 to 103 per cent. Since the means of logarithms are geometric, they are less than arithmetic means. The latter are given in the last column of Table III B, and these values are necessarily used for determining fresh spore weights.

STABILITY OF SPORE SIZES

It is commonly recognized that the spores of a given species may fluctuate within limits in mean size depending on such factors as strain, culture medium, length of time in culture and so on. Since mean spore volumes may be used to determine fresh weights of spores it is desirable to know the degree of constancy in these values. The data herein reported have been obtained over a long period of time and in some cases there are available statistics on populations obtained under different circumstances.

Monilinia fructicola. Seven different lots of 100 conidia of *M. fructicola* which were measured with similar precision had been obtained by different methods at different times. These methods included a comparison of conidia freshly collected by rubbing the agar slant, which was flooded with water and then washed and centrifuged in the usual manner (13), with an aliquot of the same spores which had been allowed to stand in water for 20 hours. Also available were conidia which had been collected dry by means of a small aluminum cyclone collector[7] (20) and which subsequently remained unwashed or were washed and centrifuged. These data are summarized in Table IV. The significant difference between means successive ranks apart determined by the method of Hartley as described by Snedecor (18, p. 253) are given at the foot of the table. For example, samples B and C rank respectively first and seventh in volume size. The difference required for samples six ranks apart is 63 and since this is exceeded the two samples differ significantly in volume size. The point of interest is that the different lots of conidia are not homogenous and that the heterogeneity although not pronounced is statistically significant and appears to be associated with spores raised and collected at different times rather than with the method of collecting. Thus comparisons of spores from the same agar slant which were either measured directly or allowed to stand in water, i.e., A versus B; or were unwashed or washed, i.e., D versus E;

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[7] This model of collector was developed by the late Dr. C. V. Lowther of the Plant Industry Station, Beltsville, Maryland, and several were kindly furnished by Dr. H. A. Rodenhiser.

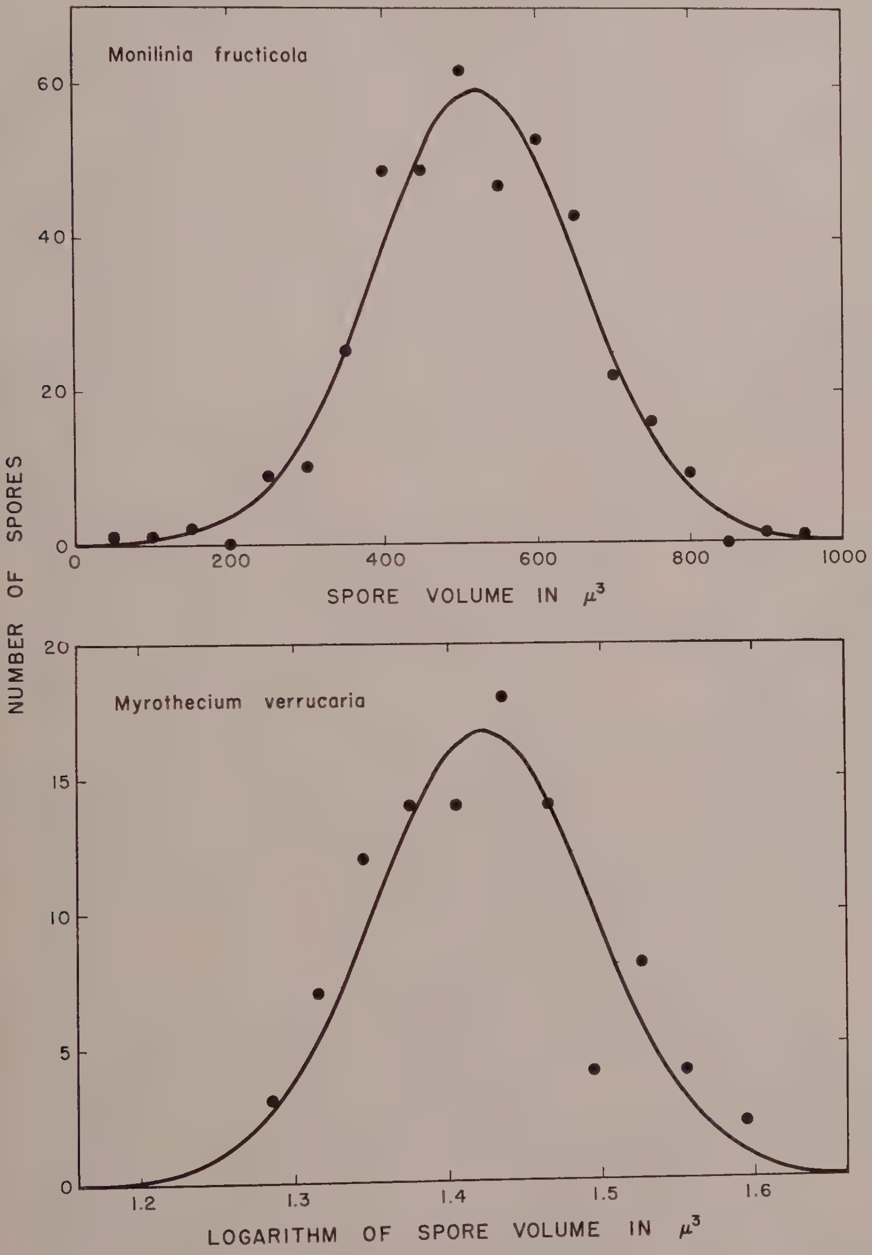

Figure 3. Typical distribution curves for spore volumes normally distributed (*Monilinia fructicola*) and log-normally distributed (*Myrothecium verrucaria*).

TABLE III

Size Distribution of Spore Volumes of 24 Species of Fungi with Means and Standard Deviations

A. Spore volumes normally distributed

Species	Mean, μ^3	Standard deviation (s)	Coefficient of variation
Uromyces caryophyllinus†	15,600	3,000	19
Stemphylium sarcinaeforme	8,680	3,460	40
Puccinia antirrhini†	6,180	945	15
Tilletia caries	3,730	510	14
Venturia inaequalis	616	201	33
Botrytis cinerea	556	212	38
Venturia pyrina	546	214	39
Monilinia fructicola	523	134	26
Ustilago maydis	259	51	20

B. Spore volumes log-normally distributed

Species	Log. volume μ^3		Antilog. volume μ^3		Arithmetic mean μ^3
	Mean	s	Mean	s	
Alternaria solani	3.969	0.162	9,310	1.45×	9,960
Erysiphe polygoni	3.871	0.077	7,430	1.19×	7,550
Uromyces phaseoli	3.782	0.057	6,050	1.14×	6,110
Phytophthora infestans	3.707	0.139	5,090	1.38×	5,360
Alternaria oleracea	2.659	0.178	456	1.51×	497
Pestalotia stellata†	2.486	0.139	306	1.38×	321
Glomerella cingulata	2.438	0.159	274	1.44×	292
Rhizopus nigricans	2.427	0.279	267	1.90×	323
Neurospora sitophila†	2.357	0.307	228	2.03×	283
Neurospora crassa	1.888	0.306	77.3	2.02×	99.3
Aspergillus niger†	1.430	0.181	26.9	1.52×	29.2
Myrothecium verrucaria	1.423	0.071	26.5	1.18×	26.8
Penicillium expansum	1.213	0.073	16.3	1.17×	16.6
Phyllosticta sp.	1.023	0.154	10.5	1.43×	11.2
Cephalosporium acremonium	0.643	0.233	4.4	1.71×	5.11

†=For qualifications see Table II and text.

TABLE IV

Mean Spore Volumes of Conidia of Monilinia fructicola Collected by Different Methods. 100 Spores per Sample

Sample	Method of collecting	Mean volume μ^3
A	Rubbed from agar slant, washed and centrifuged	580
B	Ditto, allowed to stand in water 20 hours	613
C	Nonwet, cyclone collected	486
D	Ditto, nonwashed	522
E	Ditto, washed and centrifuged	519
F	Rubbed from agar slant, washed and centrifuged	542
G	Ditto, but one day older	512
Significant differences for means, successive ranks apart:		42, 50, 55, 58, 61, 63

or were a day older, i.e., F versus G, did not differ. The four samples indicating a single homogenous population, namely D, E, F, and G were used for the distribution study reported above. Another example of spores from a different source are those reported earlier (10, 15) which had mean volumes respectively of 718 and 742 cubic μ. These values were actually obtained from linear measurements made in 1930 (22). The former mean volume was based on the volume calculated for the spore of mean linear dimension, the latter from the volumes of all individual spores. The present strain of *M. fructicola* was isolated from a naturally infected cherry in 1938 (13) and has been maintained on potato dextrose agar ever since. While this reduction in size may be associated with length of time in culture, it should be pointed out that in the earlier measurements somewhat less attention was directed toward an unbiased method of selecting the spores for measurement. The spores reported in Table II were obtained over a four-month period.

Aspergillus niger. The three lots of *A. niger* conidia were obtained over a three-year period and constituted a homogenous population. Included here is one lot which possibly was from another strain than that indicated.

Neurospora sitophila. The three lots of *N. sitophila* conidia obtained and measured over a four-month period were also homogenous as determined by an analysis of the logarithms of the volumes. This strain was isolated from a contaminated culture prior to 1936 (12) and possibly was the first use of a *Neurospora* in biochemical studies. The stock cultures have been maintained continuously on potato dextrose agar.

Neurospora crassa. The measurements reported are on Dodge Strain 6; similar measurements made on Dodge Strain 16 (15), not reported above, gave a mean value for the logarithm of the volume of 1.87 and arithmetic mean of 86.3 cubic μ (15). These values did not differ significantly from the comparable ones for Dodge Strain 6 discussed above.

Rhizopus nigricans. Another isolate of *R. nigricans* which had been maintained on potato dextrose agar culture for 14 years as previously reported (10, 15) had a mean volume of 51.9 cubic μ. This value is markedly less than the more typical value of 322.8 cubic μ, reported above, and presumably was due to continued culturing on a low nutrient medium (15, p. 153). The earlier values were based on measurements made on three lots of 100 spores over a period of three months. The three lot means differed by less than 5 per cent from their mean of 51.9 cubic μ, and also had a log-normal distribution.

Glomerella cingulata. The data of 1931 (11) on 50 spores of this species indicated that the shape of the spores was approximately that of a cylinder with hemispherical ends. However a majority of the present spores[8] appeared to be prolate sphaeroids, though some could be classified as above. The spores from the earlier data were reported (15) to have arithmetic mean volumes of 262 cubic μ. Spores identified as cylinders with hemispherical ends would tend to be about 30 per cent larger than those measured as prolate sphaeroids. Actually the difference would exceed this for the long thin spores and would be less for the short thick spores. The value of 262 reduced by about 30 per cent would be significantly different from the arithmetic mean of 292 reported in Table III on recently measured spores. However the isolates were not the same. The earlier data on only 50 spores also indicated a normal distribution.

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[8] Culture kindly furnished by Dr. Jack Ziffer, Pabst Laboratories, Milwaukee, Wisconsin.

Alternaria oleracea. Measurements of 50 spores of *A. oleracea* reported in 1953 (15) but actually made in 1941 gave arithmetic mean volumes of 653 cubic μ. This would be significantly greater than the comparable value of 497 in Table I. Similar comments can be made, as under *Monilinia fructicola* above, regarding possible change in culture, though the difference is not great.

Tilletia caries. Two additional sets of measurements were made on the same lot of spores and volumes of 3810 and 3630 μ^3 were obtained. These do not differ significantly from the value reported in Table III.

COMPARISON OF SPORE WEIGHTS DERIVED FROM SPORE VOLUMES WITH THOSE OBTAINED DIRECTLY

With most fungus spores produced in culture it is of course not practical to obtain the fresh weights directly. However, a number of collections were carefully made in the cyclone collector of spores of *Aspergillus niger*, *Monilinia fructicola*, and *Neurospora sitophila* and the total number of spores and weight were determined directly. The weights of the spores as calculated from the spore volumes using a density factor of 1.1 were compared with the direct weights. The results given in Table V show that there is considerable variation and that the weights derived from the volumes tend to run high for *M. fructicola* and *N. sitophila*; however, the differences were not significant.

TABLE V

COMPARISON OF SPORE WEIGHTS DERIVED FROM SPORE VOLUMES (DENSITY 1.1) WITH THOSE OBTAINED DIRECTLY

Species	$\frac{\text{Volume weight}}{\text{Direct weight}}$	Standard deviation
Aspergillus niger	1.08	0.37
Monilinia fructicola	1.49	0.33
Neurospora sitophila	1.35	0.36
Tilletia caries	1.81†	0.16
Ustilago maydis	0.93	0.08
Uromyces phaseoli	1.37	0.03

† = See text for qualification.

Similar comparisons were made with spores of *Uromyces phaseoli*, *Tilletia caries*, and *Ustilago maydis*. The spores of most rust and smut fungi of course are readily obtained and weighed. As far as possible all extraneous matter was removed before weighing and when making the spore counts clumps of spores were more or less broken up by agitating in the presence of a wetting agent. The results are given also in Table V. The agreement with the corn smut spores was good, while with the bean rust spores the weights derived from the volume determinations were somewhat high and with the smut spores decidedly high. The discrepancy with the smut spores can be partially explained by the reticulate surface of spores of *Tilletia caries*. The linear measurements as given in Table I are based on over-all dimensions. According to Fischer (3) the reticulations may be as much as 0.5 to 1.2 μ deep. The author would agree with these measurements. A double deduction of the mean depth of 0.85 μ from the mean length and width would give a calculated volume of 2810 μ^3. On this basis the ratio of weight derived from volume to that obtained directly would be 1.36. It is therefore probable that the mean volume of 3730 μ^3 as given for *T. caries* in Table III is somewhat too high. The spores of *T. caries*, however, are the only ones among the 24 species studied that have reticulations and in which this type of error would be introduced.

DISCUSSION

The division of fungus species into two kinds of distribution for spore volume sizes appears to be associated in part with the mean spore sizes so that most of the smaller-spored species are log-normally distributed. However, the larger-spored species appear in both types of distribution though somewhat more numerous in the normal distribution. The greater range in spore sizes, as shown by the coefficients of variation, also appear to be associated with the smaller-spored species.

It should be borne in mind that if the volumes of the spores are normally or log-normally distributed the linear measurements cannot also be likewise distributed; however, the spore weights could be. Bliss and Calhoun (1) have discussed a number of log-normal distributions in various biological systems.

It is probably significant that the species belonging to the same genera tend to be distributed in a similar manner. Thus the spore volumes of *Venturia inaequalis* and *V. pyrina* are normally distributed as are those of *Uromyces phaseoli* and with qualification those of *U. caryophyllinus*. On the other hand the spores of *Alternaria oleracea* and *A. solani* are log-normally distributed as is *Neurospora crassa* while *N. sitophila* tends to a log-normal distribution. A survey of other taxonomic or morphological relations, particularly with reference to manner of bearing spores, did not disclose any association with the two types of distribution.

When determining the spore volumes, the possibility of swelling was kept in mind; in fact the swelling response of *M. verrucaria* spores has been used as a measure of toxicity by Mandels and Darby (14). There was no evidence that these spores swelled in the absence of nutrients. This is further indicated by the very small range in spore volumes. Likewise as shown in Table IV, there was no indication that the conidia of *Monilinia fructicola* swelled appreciably when held in water under the conditions studied. Yarwood has reported (23) that the conidia of this species increased 146 per cent during germination. Conidia in the present studies do not germinate because of the lack of sufficient nutrients and oxygen. It is interesting to note that Yarwood has also defined *Monilinia fructicola* and the other spores he examined as prolate sphaeroids.

The stability of spore sizes as considered in this paper are based on spores supposedly produced under similar conditions. It is expected, as commonly known, that the spore dimensions of some species will change as the nature of the nutrient is varied. The stability of spore volume measurements appears dependent on the species and various other factors. While linear measurements may vary but little in different lots of spores, small changes here will be reflected in a cubing for the determination of the volume. Hence for precise studies which may extend over a long time or over relatively diverse conditions, it would be advisable to make a redetermination of the spore volumes, unless the species is known to be unusually stable.

It is recognized that the prolate sphaeroid will not completely characterize all the spores encountered in the 22 species, nor will the other shapes applied to the spores of the *Alternaria* species, largely because of irregularities in the shape of a small number of the spores. There seems to be no practical method of correcting this other than visual adjustments when making the original measurements. In any case it is not believed that this contributes significantly to the errors in determining mean spore volumes.

New data have not been presented on the specific gravities of spores, which it is necessary to use in conjunction with spore volumes, in order to obtain fresh weights. Earlier measurements, following the original method of Buller (2) have been made on various species and it has been found (11, 15) that the values do not depart appreciably from 1.1. Since the variation here is so much less than that resulting in obtaining spore volume the need for more precision in specific gravities is unnecessary.

The comparison of spore weights derived from volume measurements with those obtained from direct weighings gave good agreement with the spores of *Aspergillus niger* and *Ustilago maydis* but the ratios were high for *Monilinia fructicola*, *Neurospora sitophila*, *Tilletia caries* (as corrected for reticulations) and *Uromyces phaseoli*. It is probably significant that the spores of the first two species are more regularly shaped than those of the latter four species. This would indicate the partial inadequacy of defining a somewhat irregularly shaped body as a precise geometric figure such as the prolate sphaeroid. Also errors in linear measurements are increased approximately 3-fold when calculating volumes; thus a 10 per cent linear error would result in an error in volume of 30 per cent. A possible explanation for the lower direct weights of the spores is that on suspension in water they immediately imbibe and thereby increase their size and weight. However, there is no indication as pointed out above that any appreciable swelling occurs after the measurements have begun.

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IMPROVEMENTS IN CULTURING LARVAE OF AEDES AEGYPTI (L.) FOR USE IN INSECTICIDE BIOASSAY

Albert Hartzell, H. P. Burchfield, and Val F. Cotty[1]

SUMMARY

The present publication presents some improvements of a method previously described (1, 2, 3) for rearing larvae of *Aedes aegypti* (L.) for use in insecticide bioassay. The principal changes include the use of deionized water, the incorporation of physiological salt mixture in the rearing medium for test larvae, adjustment of the temperature to 31° C., and changes in the ratios of the constituents of the nutrient. Under these conditions second instar larvae are obtained with great regularity 22 hours after the addition of the nutrient to the medium. This eliminates the need for segregating out first instar larvae and thus results in considerable savings in time and effort.

Burchfield *et al.* (2) have described a method of insect bioassay based on photomigration of mosquito larvae, *Aedes aegypti* (L.). Methods for rearing test larvae were described (3) but several inherent difficulties still remained such as the time required to maintain the stock culture, the unnecessarily high concentrations of certain constituents in the rearing medium, and the fluctuation in the supply of larvae for testing. The present paper presents several changes in the procedure which have made it possible to overcome these difficulties.

PREPARATION OF NUTRIENT

The stock nutrient for rearing culture and test larvae was prepared by mixing 50 grams of brewer's yeast (U. S. P. XII, supplied by Nutritional Biochemicals Co.), 15 grams of blood albumen (Fisher Scientific Co.), and 10 grams of granular sucrose. The mixture was blended thoroughly and stored under refrigeration in a screw top bottle. On the days that the medium was required, 2.5 grams of the dry mixture were placed in a mortar, ground to a smooth paste with 10 ml. of deionized water and diluted to 1 liter. Aliquots of the agitated suspension were added to the flask containing the larvae. Deionized water for rearing the test larvae was obtained by passing once distilled water through a Barnstead "Bantam" Demineralizer. The water was tested as it passed through the resin column and maintained at less than 0.5 p.p.m. solids, calculated as NaCl. This was done so that the rearing medium could be reconstituted to a standard ionic strength by adding physiological salt.

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[1] Present address: Bristol-Myers Products Division, Research Laboratories, Hillside, New Jersey.

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HATCHING

Pyrex test tubes plugged with cotton, containing 10 ml. of 0.15 per cent U. S. P. XIV salt mixture and 1 per cent dextrose, were heated in a water bath for 5 minutes to remove dissolved oxygen. The tubes were cooled to 33° C. and strips of paper toweling containing moist 5- to 10-day-old eggs were placed in them for 20 to 30 minutes to allow hatching. The larvae were poured off into an open Petri dish containing approximately 100 ml. of deionized water. If the larvae are in contact with the salt mixture for more than 30 minutes, irregular growth and high morbidity result.

REARING LARVAE FOR TESTING

Two thousand larvae were counted with the aid of a hand tally counter and transferred with a medicine dropper to 8 liters of the rearing solution in a 12-liter round bottom flask. The rearing solution was prepared as follows: 800 mg. of U. S. P. XIV salt mixture were added to 500 ml. of deionized water heated to 45° C. for 5 minutes. The solution cooled to room temperature was adjusted to pH 6, with $1N$ HCl and $1.25N$ NaOH, the pH was determined with "Alkacid Test Paper." The salt solution was added to 7.5 liters of deionized water containing 4 ml. of a 1 per cent aqueous Pluronic F-68 solution (a surface active agent supplied by Wyandotte Chemical Co.). The flask containing this mixture was placed in a water bath held at 31°± 0.5° C., and upon equilibration to temperature, the 2000 hatched larvae were added to the flask which was then stoppered with a cotton plug. After 18.5 hours, 225 ml. of freshly prepared nutrient suspension were added. Following 22 hours of incubation the larvae were removed from the rearing solution by screening through a 60-mesh sieve set in a shallow pan arranged so it could overflow without exposing the larvae to air. They were then washed with a 10 p.p.m. solution of Pluronic F-68 (the wetting agent) into a white porcelain pan. The pan was then placed so that one end was under a fluorescent light and the larvae were given several minutes to migrate to the opposite end. The larvae that migrated to the darker end were collected with a medicine dropper and placed in a beaker containing a 10 p.p.m. solution of Pluronic F-68.

TESTING METHODS

Aliquots of the wetting agent solution containing the larvae were placed in a partly filled open Petri dish. Groups of 100 larvae were counted out and placed in 50 ml. of deionized water. The larvae in 50 ml. of solution were poured into a 250-ml. Erlenmeyer flask and 50 ml. of water solution containing the test insecticide were added over a 30-second period. The exposure time of the larvae to the insecticide varied with the speed of action of the insecticide. Rapid-acting insecticides such as pyrethrum and DDT required short exposures (20 min. to 5 hours) while slower acting insecticides such as Malathion and Diazinon required long exposures (18 to 24 hours). Short term tests permit the use of 1 ml. of insecticide in acetone, ethanol or Carbitol (diethyleneglycol mono ethyl ether) while long term tests limit the quantity of solvent used to 0.5 ml. Higher concentrations of solvent induce morbidity greater than in the untreated checks. Aside from the changes in the procedure listed here the method of testing is as stated in a previous publication by Burchfield, Redder, Storrs and Hilchey (3).

REARING CULTURE LARVAE

In establishing and maintaining an adult colony, eggs were hatched as previously described and groups of 250 larvae were placed in 2-liter flasks containing 1 liter of distilled water and the nutrient suspension. The flasks were stoppered with cotton and then incubated at 31° C. in a thermostatically controlled, warm air cabinet. As the larvae grow, their need for nutrients increases. Contaminants also build up within the flask. With these factors in mind the following time schedule was established.

First day: 250 larvae were placed in a 2-liter Erlenmeyer flask containing 1 liter of distilled water and 50 ml. of nutrient suspension. The flasks were then transferred to the incubation chamber.

Third day: the larvae were separated from the culture medium with the aid of a 60-mesh sieve and placed in another flask containing 1 liter of distilled water and 100 ml. of nutrient suspension. The flask was then returned to the incubation chamber.

Fifth day: by this time practically all of the larvae were in the fourth instar with the exception of a few individuals which had pupated. The larvae and pupae again were separated from the rearing medium with the aid of a 60-mesh sieve and transferred to a one-pint Mason jar containing 180 ml. of distilled water and 50 ml. of nutrient suspension. Several small corks were floated on the surface of the medium to support the newly emerged adults. Cheesecloth was placed over the top of the jar and held in place by the open ring type screw caps such as are used with the Mason jars. The jars were returned to the incubator and the adults emerged during the sixth, seventh and eighth days. Each day the adults were released into the culture cage housing the colony.

MAINTAINING AN ADULT COLONY

The adult colony was maintained in a 30×30×30-inch wooden cage. The front was provided with a 23×11-inch glass window, and a 10×13-inch opening covered by a muslin sleeve to provide for light, ventilation, and the transfer of materials.

The adults were fed a 1.25 per cent dextrose solution contained in a 125-ml. Erlenmeyer flask supplied with a cheesecloth wick. Two guinea pigs held in place on wooden blocks were introduced into each cage for one hour daily to supply the blood meal required by the female mosquitoes. Ten guinea pigs were used in rotation. Each animal was clipped and introduced into a cage every fifth day.

Strips of paper toweling (Scotts Soft Tuff) 1.5 inches wide were placed so as to line the sides of a 250-ml. beaker half full of distilled water with the edge of the paper immersed in the water.

One beaker was placed in each cage daily. The mosquitoes deposited their eggs on the paper toweling near the surface of the water. The strips containing the eggs were collected at 24-hour intervals and washed free from debris. They were then stored between moist filter paper in a closed container for five days. The relative humidity should not be allowed to fall below 75 per cent for the first 5 days in order to insure viable eggs. The egg strips and filter papers must be checked daily to see that approximately this relative humidity is maintained and that no mold develops. Moldy filter paper should be replaced with fresh moist filter paper. After this period the eggs were air dried for more permanent storage. The desiccated eggs were kept as a reserve supply in the event of failure of the adult colony.

REPRODUCIBILITY OF IMPROVED METHOD

In order to test the reproducibility of the improved method described in this paper, groups of 100 second instar larvae were exposed in parallel tests on six different days to a preparation of pyrethrum that had been previously partially degraded by exposure to light. The photomigration method of testing was used. The concentration at the start was 2 micrograms per 100 ml. containing 100 larvae and the exposure time was one hour. There was no significant difference between tests. An analysis of variance (4) showed that the inconsistency of times on the six days is no greater than would be expected from the theoretical binomial variance for samples of 100 insects classified as moribund or normal. The theoretical binomial variance in terms of equivalent angles was 81.2, while the actual variance was 79.3.

From this it follows that the improved method yields uniform larvae that give reproducible results. The larvae reared under these conditions are no more sensitive than those previously reared, but the specific advantage of this method is that there is no interruption in the supply of uniform larvae because the variation in the laboratory stock supply of distilled water has been eliminated. In addition, all the larvae have reached second instar at the end of the 22-hour incubation period with the nutrient. Formerly it was necessary to separate out the second instar test larvae manually, whereas now all individuals have reached this stage, if care has been taken to prevent viable eggs from being introduced accidentally when the larvae were initially counted.

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SENESCENCE OF ROSES. II. DARK FIXATION OF CO_2 BY CUT BETTER TIMES ROSES AT DIFFERENT STAGES OF SENESCENCE[1]

LEONARD H. WEINSTEIN AND HENRY J. LAURENCOT, JR.

SUMMARY

Experiments were carried out to study the amount of CO_2 fixed in the dark by cut Better Times roses at different ages. Results show that incorporation of C^{14} into petal proteins decreased as the roses aged, while increased incorporation was found in receptacle proteins. Alpha-Ketoglutarate became labeled, and the amount of radioactivity decreased with age. No radioactivity was detected in pyruvate, which was present in small quantities.

In both petal and receptacle tissues, radioactivity was detected in malate, citrate, succinate, and fumarate. In petals, the amount of radioactivity in malate remained relatively constant, while it fell off in citrate. In receptacles, there was an increase in labeled malate and a decrease in labeled citrate during the aging process.

Total glucose, fructose, ribose, and xylose were detected in petal tissues. Both glucose and fructose decreased while ribose and xylose increased in concentration during aging. Insignificant radioactivity was detected in the sugar fraction.

In petals, significant radioactivity was detected only in aspartate, glutamate, and glutamine with a trace present in alanine, serine, glycine, and asparagine. In receptacles, activity was detected in aspartate, glutamate, glutamine, serine, asparagine, and glycine, with traces in alanine, valine, γ-aminobutyrate, and the leucines. In petals, the amount of C^{14} incorporated into aspartate, glutamate, and glutamine decreased with age of tissue. In general, the opposite was true for amino acids and amides in receptacles, with the exception of serine.

The molar ratios of amino acids of petal and receptacle proteins at different ages show no significant differences, with the exception of aspartate and proline; nor is much qualitative difference evident between petal and receptacle proteins.

The total amount of C^{14} incorporated into petal tissues decreased during aging. There was a net increase in the total amount incorporated into receptacle tissues. On a total bloom basis, there was a small increase with age.

The data suggest participation of the Krebs cycle in roses. The dark fixation mechanism appears to be impaired in aging petals and stimulated in aging receptacles. Conversion of malate to citrate is inhibited in aging tissues.

INTRODUCTION

During the course of previous experiments on chemical changes associated with senescence of cut Better Times roses, it was found that the behavior of malic acid in petals and in green tissues differed considerably (26). In petals, malate was found to accumulate during the entire life of

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[1] This investigation was sponsored by a grant from Roses, Incorporated.

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the cut bloom. The opposite was true in green tissues (receptacles, leaves, and stems). The sum of total malate in the entire bloom during aging indicated that there was no net change after 0, 44, or 66 hours in water, but that there was a considerable increase at 96 hours, just prior to petal abscission. Thus, it appeared that changes in malate in the various organs were not due merely to translocation from green to petal tissues, but that there was also an increased synthesis of malate in senescent petal tissues. This could be due to stimulated dark fixation of CO_2 in these tissues.

The capacity to fix CO_2 in the dark is probably indigenous to all plant tissues. Dark fixation in succulents has been reported by many workers (1, 6, 7, 8, 15, 16, 17, 18, 22, 23, 24, 25). Saltman and Kunitake have demonstrated dark fixation in tobacco (15). It has also been demonstrated in *Xanthium* and soybean (16, 19), in spinach leaves (1), and in roots, fruits, buds, cotyledons, and leaves of several plant families (8). Several investigators (1, 8, 17) have shown the major enzyme responsible to be phosphoenolpyruvylcarboxylase.

The experiment reported herein was carried out in order to derive information on the capacity of rose tissues to fix CO_2 in the dark, and to aid in elucidating the pathways of metabolism within senescing tissues.

MATERIALS AND METHODS

Cut Better Times rose (*Rosa* sp.) blooms were grown by Mr. Paul Schneeberg, Sayville, L. I., N. Y. The roses were subjected to the normal procedures of cutting, grading, and cold storage before being shipped for the experiment. Upon receipt, the roses were placed in water in a room maintained at 70° to 75° F. Five blooms were then selected for uniformity. All but the uppermost five centimeters of the stem were removed. The stems and most of the petals were wrapped tightly in aluminum foil, and the stems were then placed in a small vial of water previously sealed into a beaker. The roses were allowed to absorb water for 30 minutes, after which the water was replaced with a solution of $Na_2C^{14}O_3$ containing 10 microcuries of radioactivity. Since rose tissues usually have a pH of about 5.5, the $Na_2C^{14}O_3$ would be converted into $NaHC^{14}O_3$ as it passed out of the xylem into contiguous tissues. This material was allowed to be absorbed through the stems for a total of 90 minutes. All of the carbonate solution was absorbed after about 30 minutes; 1-ml. aliquots of water were added when necessary to facilitate movement up the stem and to prevent wilting. At the end of the treatment period, the remainder of the stem was removed and discarded, petals were detached, and the petals and receptacles of three roses were killed in boiling 80 per cent ethanol. Petals and receptacles of the two remaining roses were quick-frozen for nucleic acid and keto acid determinations.

This harvesting procedure was repeated again after 48.0 hours (one-half open), 94.0 hours (fully open), and at 118.5 hours (senescent). The condition of roses at these stages of development has been previously described (26).

After killing the tissues in boiling 80 per cent ethanol, they were ground in a VirTis "45" homogenizer. The supernatants after centrifugation and the supernatants from four subsequent extractions were combined. Alcohol-insoluble residues were lyophilized.

Each ethanolic extract was passed through a 1 × 5-cm. column of hydrogen form Dowex 50-X8, 200–300 mesh, previously bedded in ethanol. Amino acids and amides were sorbed on the resin and were then eluted with 0.4N NH_4OH in 80 per cent ethanol and by 4N aqueous NH_4OH after the method of Plaisted (11). The eluates were dried and dissolved in appropriate quantities of 10 per cent 2-propanol.

After removal of amino compounds, the ethanolic extract was passed through a 1 × 15-cm. column of formate form Dowex 1-X10, 200–300 mesh, to remove organic acids. Following sorption, the organic acids were removed by gradient elution with formic acid according to the method of Palmer (10) and Staples (20). After titrating the acids with NaOH, tubes corresponding to each organic acid peak were combined and a small amount of hydrogen form Dowex 50 was added to remove sodium ions. The solution was filtered and then taken to dryness under a stream of warm air. The residue was made to a known volume with 80 per cent ethanol and aliquots were transferred to planchets and dried. The planchets were counted in an automatic sample changer with a gas-flow Geiger-Müller tube fitted with a micro-mil window. Other aliquots were spotted on Whatman No. 1 paper and chromatographed one-dimensionally in the solvent F mixture described by Stark, Goodban, and Owens (21). Paper chromatography served as confirmation of identification previously made by the column method.

Amino acids and amides were quantitatively determined by two-dimensional paper chromatography on Whatman No. 3 paper by the method of Porter, Margolis, and Sharp (13). Radioautograms were made by cutting two-dimensional chromatograms into four equal pieces and placing each piece with a 10- × 12-inch sheet of Kodak No-Screen Medical X-ray film for 30 days. Radioactivity of individual amino acids was determined by one-dimensional chromatography on Whatman No. 3 paper. Aliquots of the amino acids and amides were streaked in a band 2 cm. wide and 7.5 cm. from the bottom of an 18- × 22-inch sheet. Eight to 10 samples could be accommodated at one time on a single sheet. The extracts were then chromatographed in 70 per cent phenol-water (4 parts of 88 per cent phenol: 1 part of water). After drying, the sheet was cut into 3.5-cm. strips corresponding with the chromatographic run of each extract. Radioactivity on the strip was determined with a one-dimensional paper strip scanner utilizing the gas-flow Geiger-Müller tube, a rate meter, and an automatic chart-recording galvanometer. Each area of the strip corresponding to one or more radioactive amino compounds was cut out and eluted quantitatively with successive washes of 50 per cent ethanol. The combined eluate was taken to dryness and the sample was dissolved in small aliquots of water and streaked on Whatman No. 3 paper as before. The samples were chromatographed one-dimensionally in *n*-butanol-acetic acid-water (45:5:14.5). Strips 3.5 cm. wide were again cut and run on the paper strip scanner. Thus by chromatographing each extract in phenol-water and each radioactive area in *n*-butanol-acetic acid-water, every major amino acid or amide, with the exception of methionine and valine which occupy the same position, could be separated. By means of a planimeter, the area under each recorded peak was measured, and counts per minute determined by use of a standard curve prepared with known amounts of C^{14}-labeled compounds.

Protein amino acids were determined after 6*N* HCl hydrolysis (15 pounds pressure for 16 hours) of the alcohol-insoluble residue. After hydrolysis, the samples were filtered and taken to dryness several times on a steam bath to remove HCl. The amino acids were then sorbed on Dowex 50 and eluted with 4*N* aqueous NH_4OH. Quantitative estimations of amino acids were made by paper chromatography. Since incorporation of C^{14} into proteins was relatively low, aliquots were transferred to planchets and counted in order to determine total radioactivity.

Protein nitrogen was determined on aliquots of lyophilized alcohol-insoluble residue by the semi-micro Kjeldahl procedure (9, pp. 805–806).

Keto acids were determined on petal tissues by the chromatographic method of Isherwood and Niavis (4). After chromatography, the paper strips were run through the paper strip scanner, and the spots were then eluted for quantitative estimation.

Extraction of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) was carried out by the method of Howard (3). Total phosphorus of the perchloric acid extracts was determined by a reduced molybdate method (12). Sugar analyses were carried out by paper chromatography as described earlier (26).

RESULTS

Changes in concentration of protein nitrogen and amount of incorporation of C^{14} into petal and receptacle proteins are shown in Figure 1 A. Protein nitrogen was found to decrease with increasing age of rose tissues, as reported earlier (26). The capacity to incorporate C^{14} into protein amino acids diminished with age in petal tissues but increased in receptacle tissues. The apparent increase in incorporation of C^{14} into aging receptacle tissues would appear to be anomalous when viewed in conjunction with

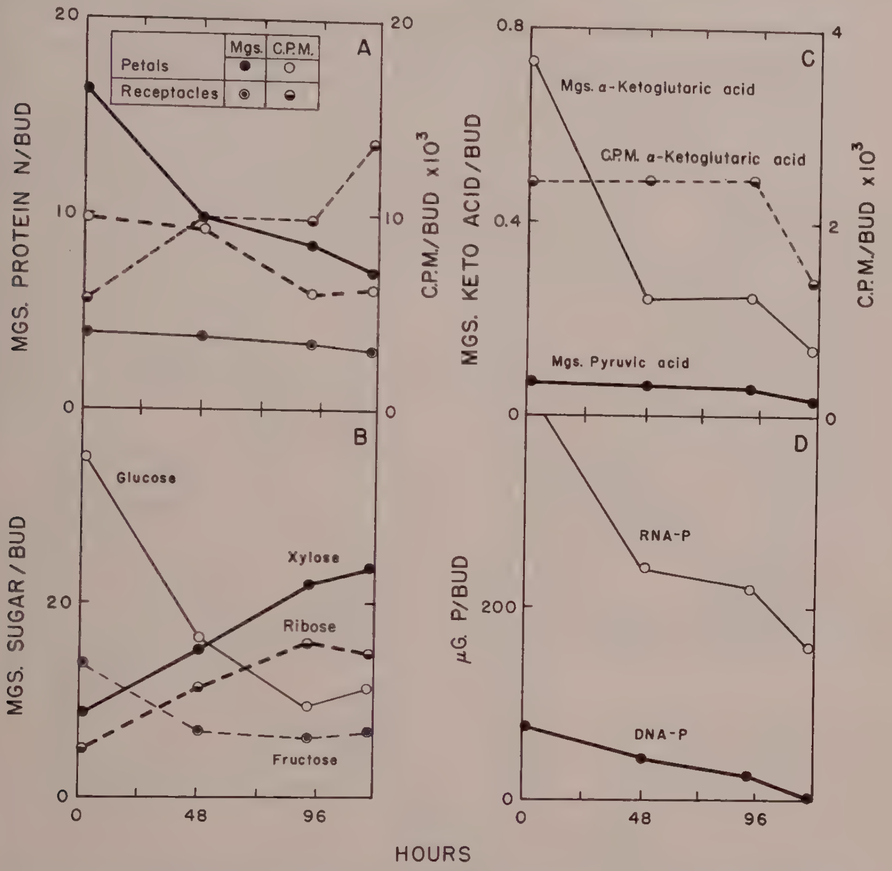

FIGURE 1. (A) Concentration and amount of C^{14}-incorporation into proteins of petal and receptacle tissues, (B) concentration of glucose, fructose, ribose, and xylose of petal tissues, (C) concentration of keto acids and amount of C^{14}-incorporation in α-ketoglutaric acid of petal tissues, and (D) concentration of ribonucleic acid- and deoxyribonucleic acid-phosphorus of petal tissues.

the decreasing protein content of these tissues; however, this discrepancy may be due to the initiation of an active synthesis of seed proteins at the expense of existing proteins in the sepals, ovary wall, etc., or since incorporation into free amino acids is greater in older receptacle tissues, this may merely reflect the utilization in the normal turnover of proteins of amino acids and amides which are more radioactive.

Changes in concentration and of incorporation of C^{14} into keto acids of petals are shown in Figure 1 C. Both α-ketoglutarate and pyruvate were lower in concentration in old than in young petals, although the latter decreased at a more uniform rate. The incorporation of C^{14} into α-ketoglutarate was also found to decrease with age, with the greatest drop occurring between the 94.0 and 118.5-hour harvests. No radioactivity was detected in pyruvate, although it was present in such small quantities that detection may have been below the threshold of sensitivity of the instruments used.

Glucose, fructose, ribose, and xylose were detected in rose petal tissues. In Figure 1 B, it is shown that both glucose and fructose are considerably reduced with age, while the pentoses, ribose and xylose increase. Insignificant incorporation was detected in the sugar fraction. Increase in pentoses was probably due to breakdown of polysaccharides or glycosides during aging.

As senescence progressed, both ribonucleic acid-phosphorus (RNA-P) and deoxyribonucleic acid-phosphorus (DNA-P) were found to fall off in petal tissues (Fig. 1 D).

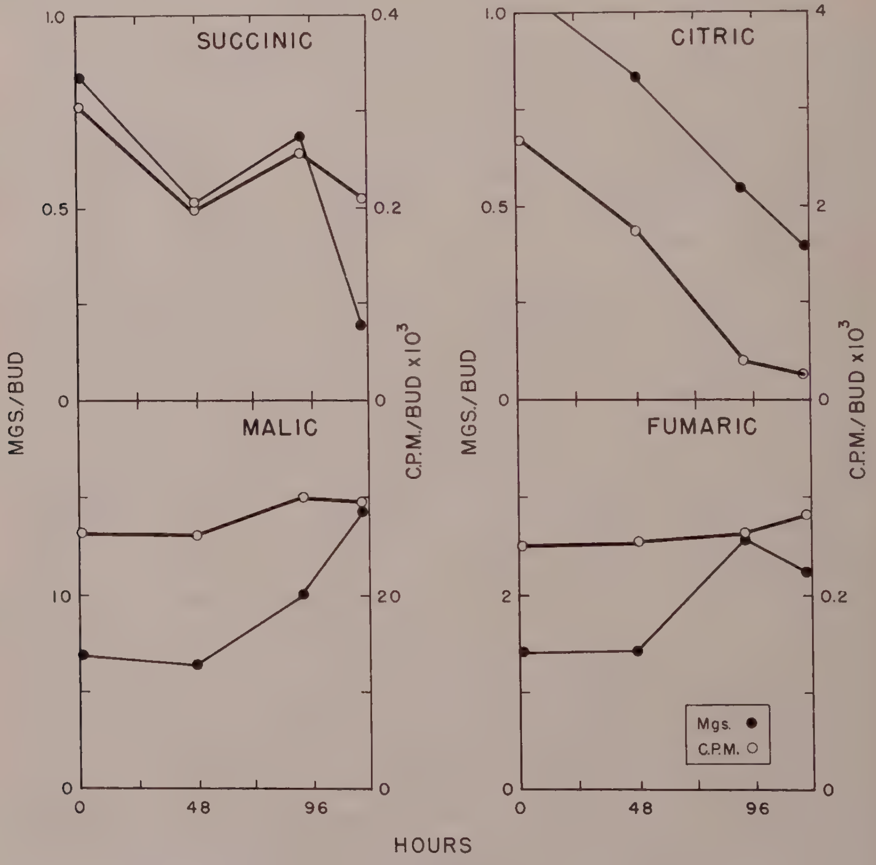

FIGURE 2. Concentration and amount of C^{14}-incorporation in organic acids of petal tissues.

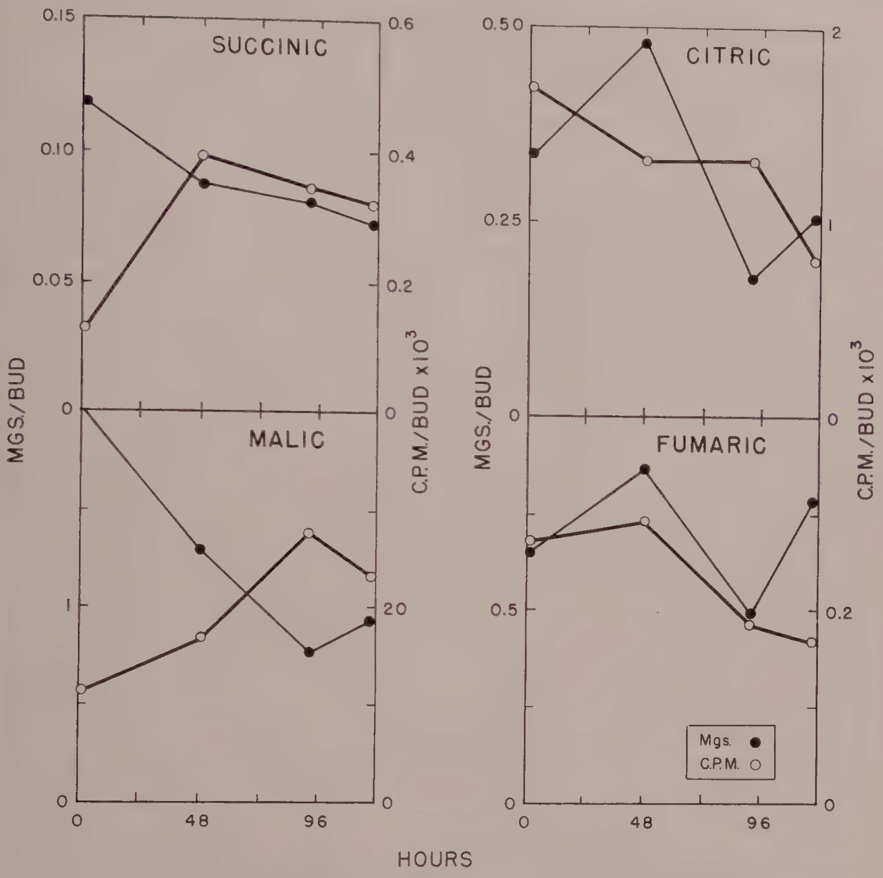

FIGURE 3. Concentration and amount of C^{14}-incorporation into nonketo organic acids of receptacle tissues.

Five organic acid peaks were eluted by ion exchange column chromatography. Of these, malic and citric acids were further identified by paper chromatography and by infrared absorption spectra. Succinic and fumaric acids were further identified by paper chromatography. The unknown peak, which is the first peak eluted from the column, was found to be composed of more than one compound, one of which has been shown to be quinic acid. Changes in concentration and amount of C^{14} incorporation into the organic acids in petals are shown in Figure 2. Both the concentration and radioactivity of succinic acid followed the same general, but somewhat erratic, pattern. The behavior of malic acid indicates a substantial increase with age, while incorporation of C^{14} remained relatively constant. In the case of citric acid, both concentration in the tissues and amount of radioactivity fell off at a uniform rate during aging. Fumaric acid increased in concentration for 94.0 hours after which it fell off slightly; the amount of incorporation into fumarate increased slightly throughout.

In the case of the green receptacle tissues (Fig. 3), the concentration of succinate fell off gradually with age, while the amount of C^{14} incorporated increased from 1.5 to 48.0 hours, and then fell off. Malate content fell off uniformly for 94.0 hours, after which there was a slight increase. Incorporation of C^{14} into malate exhibited an inverse relationship. Citrate concentration increased from 1.5 to 48.0 hours, dropped sharply between 48.0 and 94.0 hours, and increased again between 94.0 and 118.5 hours. The amount of incorporation into citrate, however, fell off gradually with time. Fumarate content followed a pattern similar to that of citrate; incorporation followed fumarate concentration for 94.0 hours, after which it continued to fall off.

As reported earlier (26), the following free amino acids and amides were detected in rose petal and receptacle tissues: aspartic acid, glutamic acid, serine, glycine, threonine, alanine, valine, γ-aminobutyric acid, proline, tyrosine, phenylalanine, the leucines, asparagine, and glutamine. In addition, lysine, arginine, and methionine sulfoxide were detected.

In petals, significant incorporation of C^{14} was present only in aspartic and glutamic acids, and in glutamine, with traces in serine, asparagine, and glycine. In receptacles, incorporation was detected in aspartic and glutamic acids, glutamine, serine, asparagine, glycine, with traces in alanine, valine, γ-aminobutyric acid, and the leucines.

In petals, the amount of C^{14} in aspartic and glutamic acids, and in glu-

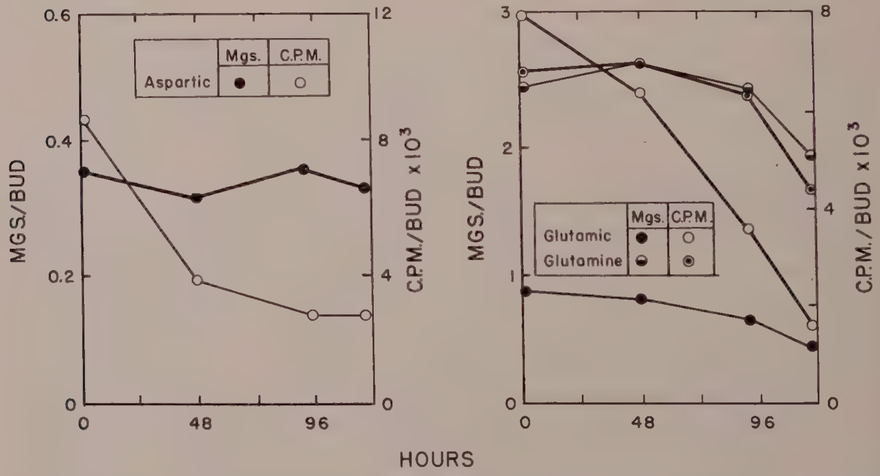

FIGURE 4. Concentration and amount of C^{14}-incorporation into amino acids of petal tissues. Small amounts of radioactivity were also detected in serine, glycine, and asparagine.

tamine decreased with time. The concentration of aspartate in these tissues during the aging process changed little, thus its pattern was quite different from that shown for incorporation. In the cases of glutamate and glutamine, however, the concentration and incorporation curves fall off with time (Fig. 4).

The patterns of changes associated with either concentration or C^{14} incorporation of amino acids or amides in receptacle tissues are shown in Figure 5. With the exception of serine, all amino acids and amides incorporated more C^{14} with increasing age. In general, the concentrations of aspartate, glutamate, serine, asparagine, and glycine decreased with time. Thus, in several instances, a pattern of increasing radioactivity and decreasing concentration has been established.

The molar ratios of amino acids of petal and receptacle proteins are shown in Table I. With the exception of aspartic acid and proline, it would appear that there were no significant changes in amino acid composition of either petal or receptacle proteins during senescence, nor is any qualitative difference evident between petal and receptacle proteins. That there are other differences, such as in protein structure, amide composition, etc., is unquestionable but cannot be determined from these data.

In Table II the total amount of C^{14} incorporated in roses at each stage of senescence is shown. These data indicate that there was a net decrease in the total amount of C^{14} incorporated (during the 90-minute treatment period) into petal tissues as they aged. There was a net increase in the total amount incorporated into receptacle tissues. On a total bloom basis, there was a small increase with age. Table III shows the amount of C^{14} incorporated into each major constituent as percentage of total incorporation. In petals, a greater percentage of total incorporation was found in the organic acid fraction, primarily at the expense of amino acids and amides. In receptacles, no such clear pattern existed.

DISCUSSION

Rose petal and receptacle tissues have an active dark CO_2 fixation mechanism. Identification of labeled malate, citrate, α-ketoglutarate, succinate, and fumarate suggests the participation of the Krebs cycle in the metabolism of these tissues. Participation of active amination reactions are indicated by the presence of labeled aspartate, glutamate, and alanine, and the identification of labeled serine and glycine suggests the reaction:

$$\text{pyruvate} \rightarrow \text{hydroxypyruvate} \rightarrow \text{serine} \rightarrow \text{glycine}.$$

There is no way of assessing the importance of upward and downward translocation of labeled compounds between petal and receptacle tissues during the 90-minute fixation period. Table II, for example, shows that the total amount of C^{14} fixed decreased steadily in progressively older

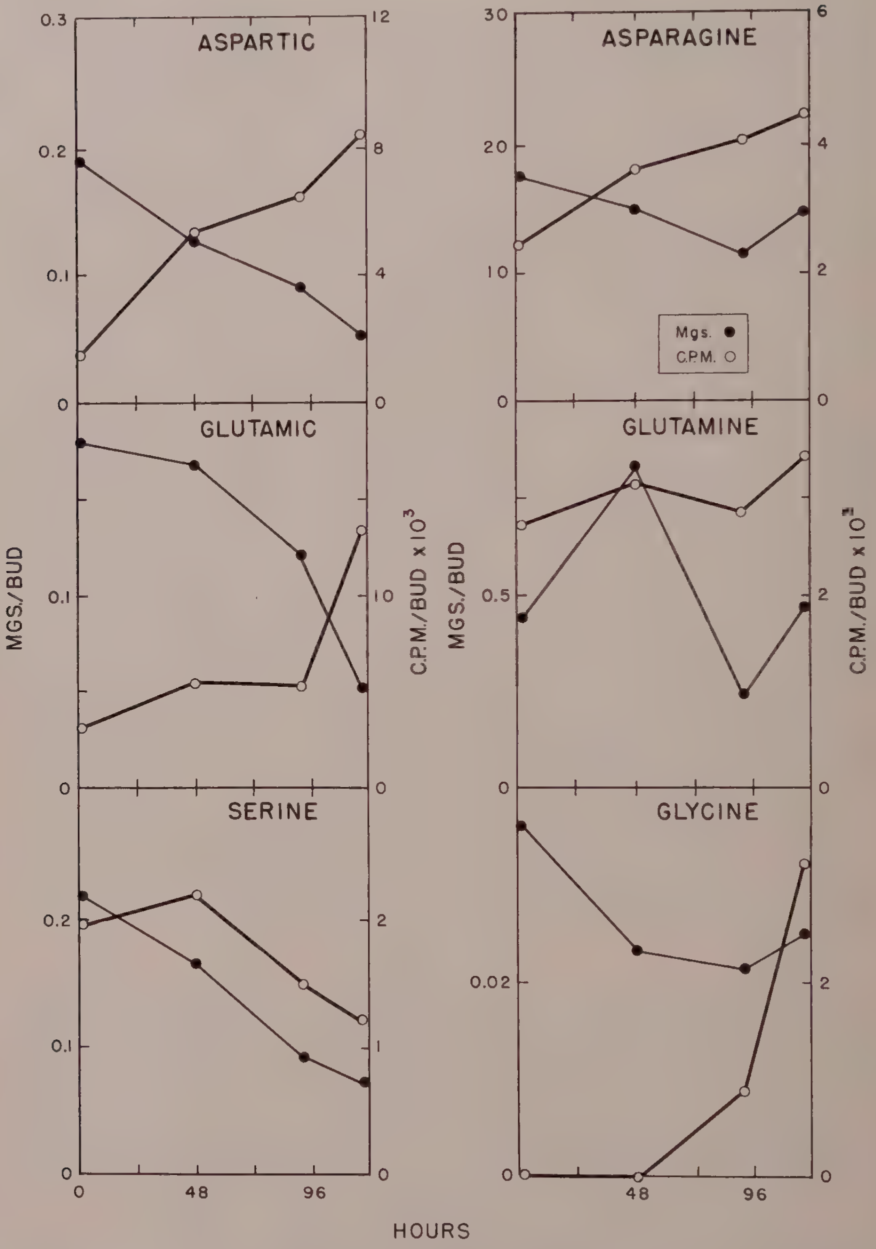

FIGURE 5. Concentration and amount of C^{14}-incorporation into amino acids of receptacle tissues. Small amounts of radioactivity were also detected in alanine, valine, leucines, and γ-aminobutyric acid.

TABLE I

Molar Ratios of Protein Amino Acids of Petal and Receptacle Tissues during Aging. Ratios Are Based on Threonine as 1.0

Acid	Molar ratios at different ages (hr.)			
	1.5	48.0	94.0	118.5
	Petals			
Aspartic	1.7	1.2	1.6	6.9
Glutamic	2.4	2.1	2.2	2.2
Serine	1.1	0.9	1.0	1.1
Glycine	1.2	1.0	1.2	1.2
Threonine	1.0	1.0	1.0	1.0
Alanine	1.5	1.3	1.5	1.4
Lysine	1.7	1.6	1.8	1.7
Arginine	1.0	1.1	0.9	1.1
Proline	0.6	0.4	0.8	0.4
Valine	1.6	1.5	1.6	1.5
Leucines	3.5	3.7	3.9	3.4
Phenylalanine	0.9	1.7	0.7	0.8
Tyrosine	0.7	0.6	0.6	0.4
Hydroxyproline	0.6	<0.3	0.3	<0.3
Methionine sulfoxide	0.6	0.4	0.3	0.4
	Receptacles			
Aspartic	1.4	1.6	1.9	2.2
Glutamic	2.2	2.4	2.2	2.4
Serine	1.1	1.1	1.1	1.1
Glycine	1.2	1.2	1.1	1.1
Threonine	1.0	1.0	1.0	1.0
Alanine	1.4	1.5	1.4	1.5
Lysine	1.6	1.6	1.4	1.5
Arginine	1.0	1.1	1.0	1.1
Proline	0.6	0.4	0.3	0.4
Valine	1.6	1.6	1.5	1.6
Leucines	3.7	3.7	3.6	3.6
Phenylalanine	1.5	1.7	1.7	1.8
Tyrosine	0.7	0.8	0.7	0.6
Hydroxyproline	<0.3	<0.3	<0.3	<0.3
Methionine sulfoxide	0.5	0.4	0.4	0.7

petal tissues. This might suggest that there was a greater movement out of older than younger petal tissues. However, the relatively unchanged amount of labeled malate throughout would indicate that translocatory movements are not of great importance in experiments of this duration. It is more likely that the activity of the dark fixation mechanism, presumably mediated by phosphoenolpyruvylcarboxylase, is somewhat impaired in petal tissues of aged cut roses. In addition, since the major reduction in radioactivity in petals occurred at the expense of free aspartate and glutamate, it would seem likely that there is a relationship between age of petal tissue and amination reactions.

In receptacles, however, it was found that total incorporation increased

TABLE II

TOTAL INCORPORATION OF C^{14} INTO DIFFERENT FRACTIONS DURING AGING

Fraction	C.p.m. at different ages (hr.)			
	1.5	48.0	94.0	118.5
Petals				
Organic acids	32,090	30,960	32,230	30,840
Free amino compounds	23,870	17,250	12,800	8,800
Protein	9,800	9,030	5,920	6,150
Total (petals)	65,760	57,240	50,950	45,790
Receptacles				
Organic acids	17,150	18,810	29,530	24,950
Free amino compounds	11,470	19,700	23,230	28,590
Protein	5,640	9,780	9,720	13,730
Total (receptacles)	34,260	48,290	62,480	67,270
Total (bloom)	100,020	105,530	113,430	113,060

in older tissues. Such a phenomenon is not felt to be in contradiction to the preceding discussion relating to petal tissues, since the tissues designated as receptacles include the developing ovules, and one might expect important changes in metabolism involving production of seed proteins, and reserve fats, carbohydrates, etc., even in cut roses.

It is also indicated that as cut rose tissues age, there is a breakdown in the capacity of the tissues to convert malate to citrate. Thus, as is shown in Figure 2, although the amount of labeling in malate remains relatively

TABLE III

INCORPORATION OF C^{14} INTO ORGANIC ACIDS, FREE AMINO COMPOUNDS, AND PROTEINS OF PETAL AND RECEPTACLE TISSUES AS PER CENT OF TOTAL RADIOACTIVITY

Fraction	% Of total C^{14} in plants at different ages (hr.)			
	1.5	48.0	94.0	118.5
Petals				
Organic acids	49	54	63	68
Free amino compounds	36	30	25	19
Protein	15	16	12	13
Receptacles				
Organic acids	50	39	47	37
Free amino compounds	34	41	37	42
Protein	16	20	16	21

constant, radioactivity in the citrate fraction falls off continuously, the ratios of malate/citrate in petals at 1.5, 48.0, 94.0, and 118.5 hours being 10, 15, 73, and 105, respectively. These results suggest a block in malic dehydrogenase or citrogenase, or a reduced amount of pyridine nucleotides or acetyl CoA in aging detached rose tissues.

Reduction in protein content and in RNA-P, as well as the striking similarity between the concentration curves (Figs. 1 A and 1 D), confirms the dependence of protein synthesis on nucleic acids (2, 5, 14).

It should be acknowledged that the technique by which C^{14} was introduced into the rose would not necessarily yield the same results as would gaseous $C^{14}O_2$. Thus, in the former case, labeling would be greatly dependent on vascular translocation, while in the latter case, the number of stomata and total surface would be of great importance.

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SENESCENCE OF ROSES. III. ISOLATION AND IDENTIFICATION OF *l*-QUINIC ACID FROM BETTER TIMES ROSES[1]

Leonard H. Weinstein, William R. Smith, and
Henry J. Laurencot, Jr.

SUMMARY

l-Quinic acid was isolated and identified from petal and receptacle tissues of Better Times roses. Preliminary data indicate that it makes up between 1 and 5 per cent of the dry weight of petal and between 5 and 8 per cent of the dry weight of receptacle tissues, and that it is the dominant nonketo organic acid of these rose tissues.

An unknown organic acid was isolated from rose petals and receptacles of rose blossoms in the course of a study on the chemical changes associated with senescence in cut roses (31). The material was first detected in an eluate from a column of Dowex 1 resin in the fraction corresponding to amino acids. The organic acid was subsequently separated from amino acids by sorption of the latter on hydrogen form Dowex 50, and by gradient elution of organic acids from formate form Dowex 1 with formic acid.

It was the first material displaced from Dowex 1. Data are presented in this paper on the physical properties of this material and the evidence for its identification as *l*-quinic acid (1,3,4,5-tetrahydroxycyclohexane carboxylic acid).

Quinic acid is of fairly wide occurrence in plants, and it has been identified from apples (13, 14, 28), pears (25, 30), peaches (1, 3), apricots (2), whortleberries (24), cranberries (4, 6, 18, 20), prunes (4, 18), grass (15), tobacco (23), potatoes (17), *Aconitum septentrionale* (16), *Illicium verum* (5), *Angelica archangelica* (27), fir needles (21), and coffee (29).

Neither the biosynthesis nor the role of quinic acid in plants is known. Hall (11) has suggested that the biogenetic origin of quinic acid may be by condensation of one mole of metasaccharonic acid with one mole of saccharic acid. He has also postulated a key position for quinic acid in the synthesis of benzenoid compounds. Davis (7, 8, 9, 10) has shown that aromatic biosynthesis in mutant strains of *E. coli* and *Neurospora* sp. may proceed when they are fed the partially saturated ring compound, shikimic acid, but that quinic acid is not an equally obligatory intermediate. Mitsuhashi and Davis (19) have demonstrated the presence of a

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[1] This investigation was aided by a grant from Roses, Incorporated.

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quinic dehydrogenase, which oxidizes quinic acid to 5-dehydroquinic acid in a mutant of *Aerobacter*. Hulme (14) has suggested that one role of quinic acid in apples may be associated with its ability to react with relatively insoluble caffeic acid, thus making the *o*-dihydroxyphenolic properties of caffeic acid more readily available in the form of chlorogenic acid.

The present report concerns the isolation and identification of *l*-quinic acid from petal and receptacle tissues of Better Times roses, with some preliminary data as to its behavior during senescence.

MATERIALS AND METHODS

Petal tissues from 100 Better Times rose (*Rosa* sp.) blooms (about 800 grams) were ground in a Waring Blendor with sufficient absolute ethanol to give a final ethanol concentration of about 80 per cent. The brei was centrifuged and the supernatant liquid was decanted. The residue was extracted twice more with 80 per cent ethanol, centrifuged each time, and the supernatants combined. Receptacle tissues (blossom with petals removed) from these blooms (about 125 grams) were handled in the same manner. The supernatant liquids were passed through 4×25 cm. columns of hydrogen form Dowex 50-X8, 20 to 50 mesh, bedded in ethanol, to remove amino acids and other cationic substances. The eluate was then passed through 1.5×30 cm. columns of formate form Dowex 1-X10, 200 to 400 mesh, also bedded in ethanol to sorb organic acids and other anionic substances. The columns were then eluted with 100 ml. of 0.7*N* formic acid followed by 100 ml. of distilled water. The eluates were taken to dryness under a stream of warm filtered air. The residue from petals was sticky and contained a large amount of anthocyanin pigment, while the residue from receptacles was also sticky, but nearly colorless.

An aliquot of this initial fractionation was passed through formate form Dowex 1-X10, 200 to 400 mesh, and acids were removed by gradient elution with 6*N* formic acid (22), in 2-ml. aliquots. The fractions were taken to dryness at 40° C. under a stream of air. Each fraction was titrated with either 0.01*N* or 0.10*N* NaOH using bromcresol green-methyl red indicator. Only one large fraction was eluted. By paper chromatography, it was found that this fraction contained several components.

A similar aliquot of the 0.7*N* formic acid fraction was sorbed on a 1×15 cm. column of acetate form Dowex 1-X10, 200 to 400 mesh, and acids were removed by gradient elution with 2.5*N* acetic acid (22). Two-ml. aliquots were collected, dried, and titrated as described earlier. Three peaks were resolved by this procedure, the second peak being by far the major component (Fig. 1).

The materials in the tubes representing the three peaks were combined and passed through columns containing appropriate amounts of hydrogen

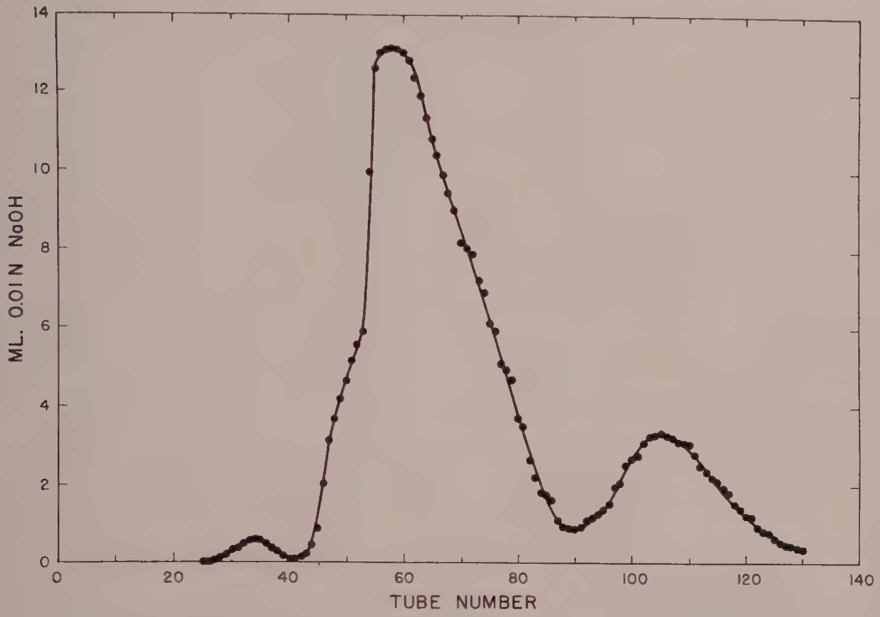

FIGURE 1. Ion exchange separation of 0.7*N* formic acid eluant from Better Times rose petals on acetate form Dowex 1-X10, 200 to 400 mesh. Eluting liquid: 2.5*N* acetic acid.

form Dowex 50-X8, to remove sodium ions and methyl red indicator. The eluates were then passed through columns of formate form Dowex 1-X10 which took up both the acids and bromcresol green. The columns were then eluted with 2 bed volumes of 1*N* formic acid and one bed volume of water. The bromcresol green remained on the column, resulting in a colorless eluate. The eluates were taken to dryness under a stream of warm, filtered air. Components of the first and third peaks did not crystallize upon drying, and were not further resolved. The component of the second peak dried down to white wax-like crystals. Infrared spectra were determined on a Perkin-Elmer Model 13 U infrared spectrophotometer in a KBr pellet (0.2 per cent unknown in KBr).

Carbon and hydrogen analyses were carried out by the Schwarzkopf Microanalytical Laboratory, Woodside, New York.

Oxidation of quinic to citric acid was carried out by the method proposed by Hulme (13). Several milligrams of the unknown were refluxed with 1 ml. of 30 per cent H_2O_2 in a boiling water bath. Aliquots were removed at 40 and at 80 minutes and chromatographed on Whatman No. 1 paper in solvent F of Stark, Goodban, and Owens (26) and in ethyl ether-formic acid-water (5:2:1) (22). Chromatography of known and of rose quinic acid was carried out in the same solvents.

RESULTS AND DISCUSSION

The unknown acid from roses had a melting point of 163° to 165° C. (uncorrected). Analyses revealed that the carbon and hydrogen contents were 43.67 and 6.55 per cent, respectively. These results suggested that the unknown was *l*-quinic acid, which has a melting point of 162° C. and a theoretical carbon and hydrogen analysis of 43.75 and 6.29 per cent, respectively. The optical properties of the unknown were determined in a 4 per cent aqueous solution. The $[\alpha]_D^{24°}$ of the rose preparation was found to be $-43.7°$. The value for *l*-quinic acid is $[\alpha]_D^{20°} = -44°$ (12, p. 297).

Paper cochromatography of authentic *l*-quinic acid and the unknown revealed that both compounds migrated to $Rf = 0.23$ in solvent F and to $Rf = 0.25$ in ethyl ether-formic acid-water solvent. Results for solvent F are shown in Figure 2 A.

Oxidation of the unknown and *l*-quinic acid with H_2O_2 indicated at least partial conversion to citric acid upon paper chromatography (Fig. 2 B).

Final proof of the identification of the unknown as *l*-quinic acid was by infrared spectroscopy as shown in Figure 3.

With positive identification of the unknown as *l*-quinic acid, it became possible to recalculate some earlier data concerning the percentage of this compound found in rose tissues. Although this work was carried out using the gradient elution method with formic acid and several small impurities are known to be present, it can be roughly estimated that *l*-quinic acid makes up between 5 and 8 per cent of the dry weight of receptacle tissues

FIGURE 2. (A) Paper chromatogram of unknown acid from roses and *l*-quinic acid run in solvent F. Left to right: *l*-quinic acid + unknown, malic acid, *l*-quinic acid, unknown. (B) Paper chromatogram of unknown acid from roses and *l*-quinic acid after oxidation in boiling H_2O_2. Chromatographed in solvent F. Left to right: *l*-quinic acid, unknown, *l*-quinic in H_2O_2 for 40 minutes, *l*-quinic in H_2O_2 for 80 minutes, unknown in H_2O_2 for 40 minutes, unknown in H_2O_2 for 80 minutes, citric acid.

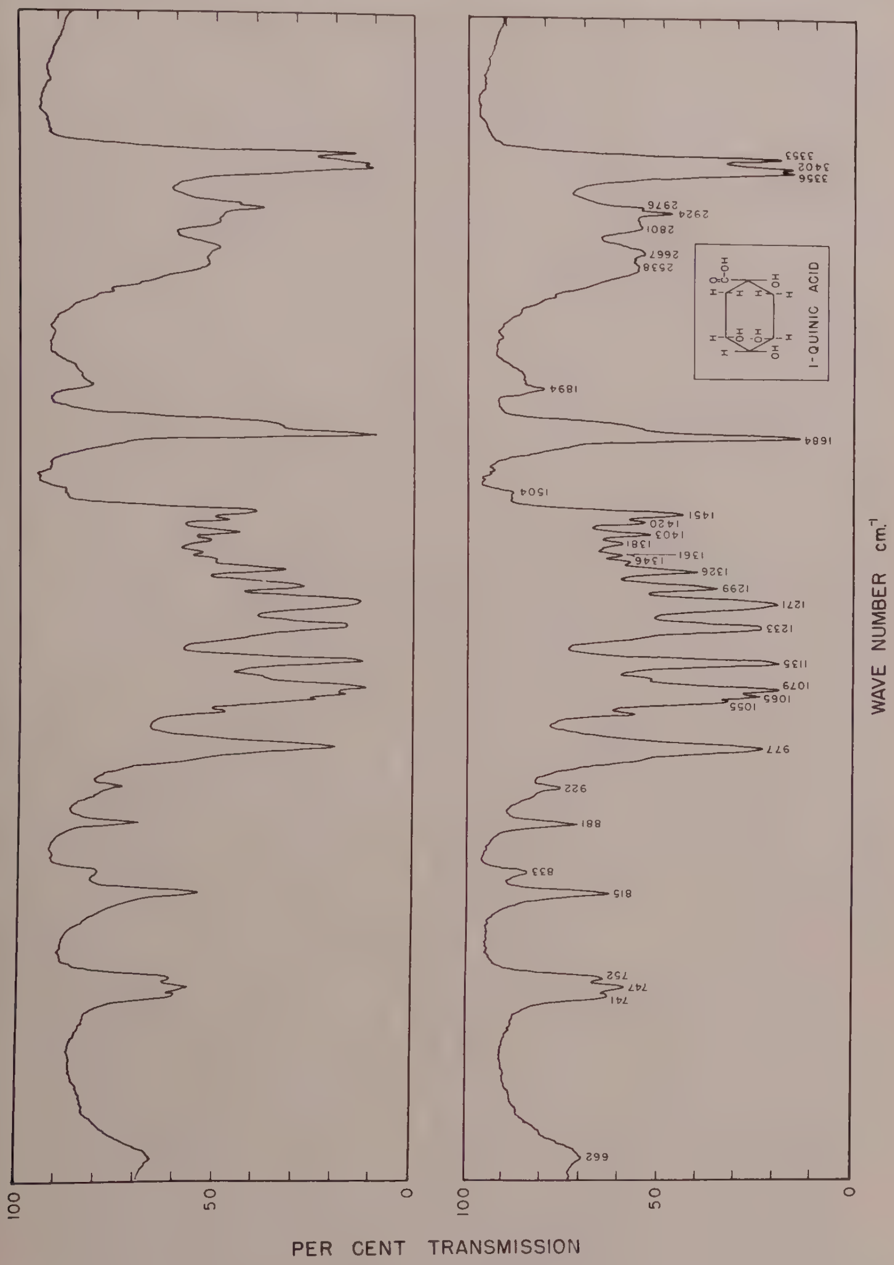

FIGURE 3. Infrared spectra of unknown acid from roses (lower) and *l*-quinic acid (upper).

of Better Times roses aged on the plants, the concentration of quinic acid decreasing with age. In petals, the same trend exists, and *l*-quinic acid makes up between 1 and 5 per cent of the dry weight.

It also becomes clear that *l*-quinic acid represents the dominant non-keto organic acid of Better Times roses, not malic acid as previously believed (31). Thus, during four stages of natural senescence, the ratios of quinic acid to malic acid in petals were found to be approximately 4:1, 3:1, 3:1, 1:1, and in receptacles, 10:1, 8:1, 7:1, 5:1. More precise experiments utilizing separation on acetate form Dowex 1 will be carried out to confirm these preliminary findings.

Quinic acid has been reported to be in highest concentration in young fruit of apples (13), peaches (1), and pears (30), but it apparently is not usually found to exceed the malate concentration. In apricots, Anet and Reynolds (2) have found that *l*-quinic makes up about 3 per cent of the total organic acidity.

Hall (11) has suggested that citric and quinic acids are not generally found in the same plants. The identification of citric acid in Better Times roses (31) and its recent isolation and crystallization from these tissues definitely establishes their simultaneous occurrence.

It is interesting to note that in a series of experiments on dark fixation of $C^{14}O_2$ by roses at different stages of senescence, no radioactivity has been detected for quinic acid. This would indicate that formation of quinic acid is not closely associated with the Krebs cycle, but more likely is involved in unassociated reactions. These data are compatible with the suggestion of Hall (11) that quinic acid is synthesized via metasaccharonic and saccharic acids from degradation of polysaccharides, since the polysaccharide fractions would not be expected to become radioactive in dark fixation experiments.

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A STUDY BY MICROAUTORADIOGRAPHY OF THE DISTRIBUTION OF TRITIUM-LABELED GLYCINE IN RUSTED PINTO BEAN LEAVES

Richard C. Staples and Myron C. Ledbetter

SUMMARY

The distribution of tritium-labeled glycine in primary Pinto bean leaves infected with the bean rust fungus was studied by microautoradiography. Rusted leaves were excised, fed glycine-2-$H^3(CH_2{}^3(NH_2)COOH)$ through the petioles, and microtome sections made of the uredinia. Much more of the radioactivity was found in the young uredospores, their sporophores and the distal portions of the mycelium than in the host. The data were interpreted to mean that an increase in glycine incorporation at the site of infection took place primarily in the fungus rather than its host.

INTRODUCTION

The early work of Gottlieb and Garner (5) clearly demonstrated that radiophosphorus moved to the site of infection in rusted wheat leaves. Later work (9, 10, 17) using C^{14}-, P^{32}-, and S^{35}-labeled organic and inorganic materials of wide variety has confirmed this localized accumulation for several rust fungi. Because the relatively powerful beta particles emitted by these tracers penetrate deeply into photographic emulsions, resolution is not good at the cellular level (14), and it has never been decided whether the tracers moved predominately into the rust fungus or host tissue at the site of infection.

Decay of tritium is completed with emission of a beta particle whose energy of radiation is of the order of 18 Kev (14). Its penetration into a photographic emulsion is very shallow, and autoradiographs with a resolution in the range of 1 micron can be obtained. For these reasons tritium has been used to study distribution of a labeled compound in rusted bean leaves at the cellular level. An amino acid was chosen, because it was hoped that it would be incorporated into the protein fraction of the host-parasite complex and thus withstand an embedding procedure capable of removing more soluble materials. Tritiated-glycine was selected as the amino acid because it was commercially available.

MATERIALS AND METHODS

Bean plants, *Phaseolus vulgaris* L. var. Pinto, were grown from seed in composted sod-soil in 4-inch pots. Ten days after planting, the primary leaves were inoculated with uredospores of the bean rust fungus, *Uromyces*

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phaseoli (Pers.) Wint., at the rate of about 0.01 mg. uredospores per leaf. This was done by mixing spores with talc (1:2 w/w), suspending the spore-talc mixture in water (2:1 w/v), and spraying the mixture onto the leaves with a DeVilbiss sprayer using 30 lb./in.^{2} air pressure. After spraying, the plants were placed overnight in a high-humidity chamber (6) at 70° F., then returned to the greenhouse bench.

When the infection had developed on the primary leaves to the point where the epidermis was ruptured and uredospores were being shed, a primary leaf was excised at the base of the petiole, the petiole immersed in 10 ml. of solution (2.5 mc./ml.) of glycine-2-H^3 of specific activity 13.0 millicurie per millimole, and the whole placed in a well-lighted hood for 24 hours. The hood was operated so that the leaf was in a slowly moving airstream to aid in transpiration. As the glycine-water solution was taken up by the leaf it was replaced by water. Approximately 1 ml. of solution was absorbed by the leaf.

After petiolar-feeding was completed, the area of the leaf containing the uredinium was excised, killed, and fixed in Craf II (8, p. 17). Following fixation, the tissue was dehydrated in dioxane (8, p. 29), embedded in "Tissuemat," sectioned on a microtome at 10 microns, and affixed to glass slides. Kodak AR-10 stripping film was applied to the sections as described by Taylor and McMaster (13). The slides were left in the dark for two weeks, developed in full strength Kodak D19b developer for 20 minutes, cleared and dried. Microautoradiographs were mounted in Shilliber's oil for photography (13).

RESULTS

In the microautoradiographs, the rust mycelia can be seen as dark, worm-like structures surrounded by a more or less speckled host tissue both in cross section (Fig. 1) and in peridermal section (Fig. 2). While both host and parasite have accumulated tritium, the latter is much more heavily labeled. Heavy labeling is indicated by a black image in the photographs. The outer portions of the mycelium can be seen to be labeled in the autoradiographs (Fig. 2 A), and this area seems to correspond to Zone III of Shaw and Samborski (10) for rusted leaves fed $C^{14}O_2$ in the light. Zone III is a ring of radioactivity away from the center of infection. Older uredospores show little activity while the younger uredospores are heavily labeled (Fig. 1 A).

Figure 2 A illustrates the radial growth of the fungus, while a comparison of Figure 1 with Figure 2 shows that growth of the fungus is mainly in a plane parallel to the surface of the bean leaf.

DISCUSSION

The classical studies of Allen and Goddard (2) and Allen (1) on powdery mildew-infected wheat leaves have made it clear that most of the

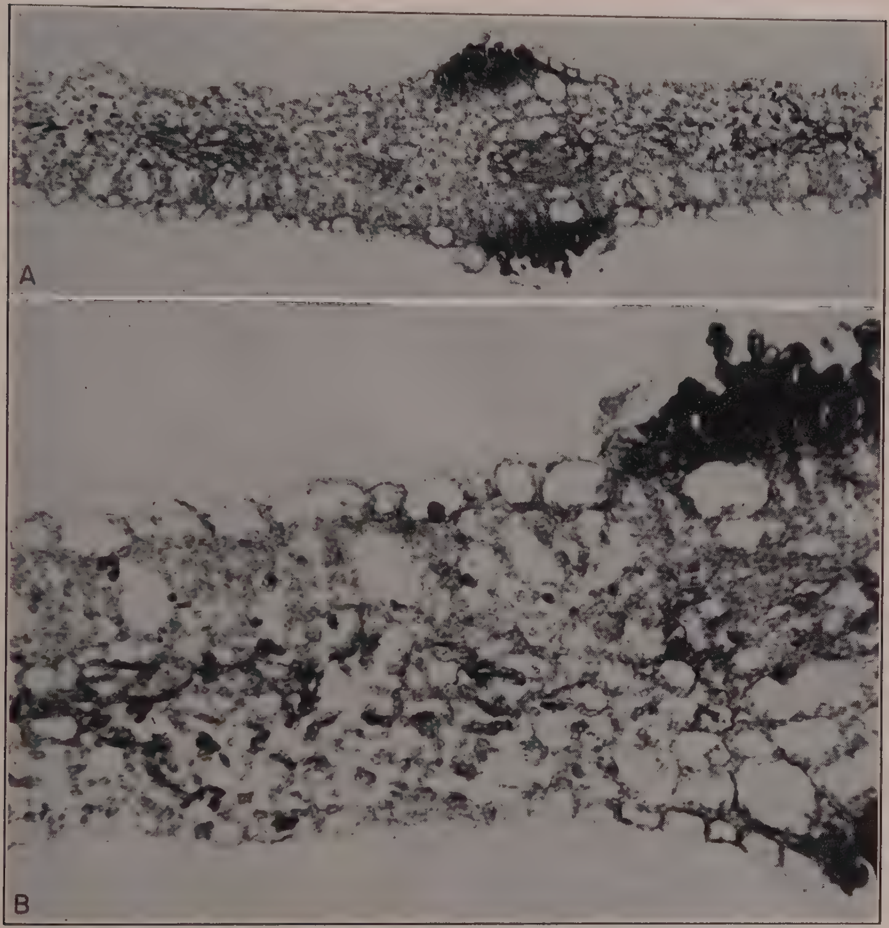

FIGURE 1. Microautoradiograph of cross section of uredinium showing relative accumulation of radioactivity between host and parasite. Dark areas indicate heavy labeling. A. ×85; B. Portion of A at ×210.

total increment of disease-induced respiration found in this host-parasite complex was contributed by the host. More recent authors (4, 7, 11, 12, 16) have extrapolated this finding to the rust fungi in interpreting their chemical and respiratory data. This has led to the view that it is the host rather than the rust fungus which contributes largely to differences which are found when the host-parasite complex is compared to the uninfected host (see for example, 11), a view supported by the work of Yarwood and Jacobson (17) for bean rust. Lately Daly, Sayre, and Pazur (3) have presented an hypothesis which suggests that, at least during later stages of development of *Puccinia carthami* on safflower, the respiration of the rust

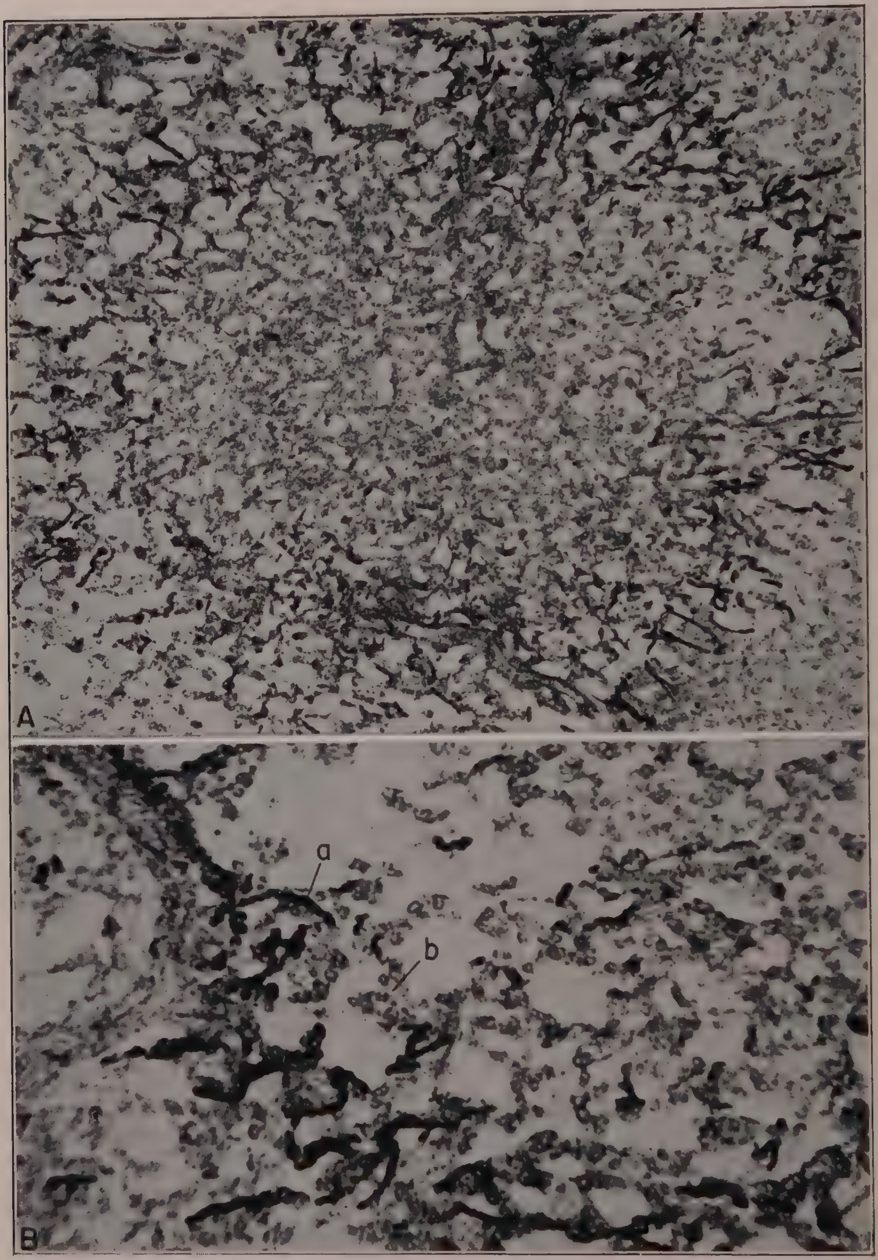

FIGURE 2. Microautoradiograph of peridermal section of uredinium showing relative accumulation of radioactivity between host and parasite. Dark areas indicate heavy labeling. A. ×100; B. Portion of A at ×250. a. Heavily labeled rust mycelium. b. Host tissue with trace of radioactivity.

fungus exceeded that of the host. If this hypothesis is true it would be less certain that chemical changes in a host brought about as the result of rust infection are occurring mainly in host cells, for it would seem that the metabolically more active fungus would behave as a "sink" (15) for materials provided by the host.

The results of the present studies indicate that most of the tritium from the glycine-2-H^3 has been incorporated into the rust-fungus mycelium rather than into host tissue though some labeling has also occurred in the latter. The heavier incorporation of tritium into young uredospores, their sporophores, and the distal portions of the fungus likely reflects the higher rate of protein synthesis in these zones, while protein turnover probably accounts for its incorporation into host tissue. No definite conclusion was reached as to the rate of fixation of tritium into the host tissue at the site of infection relative to areas more remote from the infection. However, such differences are minor in comparison to the degree of incorporation of this material into the fungus.

If there is a positive correlation between the ability of a tissue to incorporate a protein-precursor such as glycine and its rate of respiration, then the conclusions of Allen and Goddard (2) for the wheat-powdery mildew complex must not hold for the bean tissue and rust-fungus complex. In this respect Yarwood and Jacobson (17), Shaw, Brown, and Jones (9), and Shaw and Samborski (10) report greater accumulation of C^{14}- and S^{35}-labeled compounds, including amino acids, in leaf mesophyll than in mildew mycelium. These findings are in marked contrast to those reported here for bean rust, indicating a possible difference in mode of attack of the host between the ectoparasitic powdery mildew organism and endoparasitic rust fungi. Although no definite conclusions as to relative rates of respiration of host and parasite can be drawn on the basis of these studies, the parallelism between the accumulations at wheat stem rust infection sites and their respiratory rate has already been noted (7).

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COMPOSITION OF CONIDIA OF NEUROSPORA SITOPHILA[1]

ROBERT G. OWENS, HELEN M. NOVOTNY, AND MARIE MICHELS

SUMMARY

The chemical composition of *Neurospora sitophila* conidia was investigated. The percentages of major constituents were as follows: ash, 6.8; spore walls, 27.2; protein, 25.8; lipids, 17.7; ribose nucleic acid, 9.0; deoxyribose nucleic acid, 1.4; carbohydrates, 9.3; organic acids, 0.2; and free amino acids and amides, 1.3. The ash contained 28 per cent phosphorus. Phosphorus, which made up 1.91 per cent of the spore weight, was distributed among various fractions of the spores as follows (per cent): 2 per cent perchloric acid extract, 27.8 (11.5 per cent inorganic, 16.3 per cent organic); lipids, 11.1; nucleic acids, 59.6; and spore residue after removal of nucleic acids, 3.9.

The spore walls were composed of 53 per cent hemicelluloses, 32 per cent cellulose (probably in combination with other material), and about 5 per cent chitin. Ten per cent of the wall contents was unaccounted for. Bulk protein, isolated from homogenates prepared by sonic disintegration of the spores, contained 15.4 per cent nitrogen. More than 95 per cent of the lipids was phospholipids, but traces of sterols, sterol esters, fatty acids, triglycerides, and a considerable amount of carotenoids were present.

Most of the carbohydrate was not identified but glucose, hexose-phosphate, hexose-diphosphate and 3-phosphoglyceric acid were separated on chromatographic columns and tentatively identified by paper chromatography. Traces of other free sugars were present also. The following organic acids were found (μg./gram of spores): glyoxylic, 6; α-ketoglutaric, 51; oxalosuccinic, trace; pyruvic, 24; citric, 1232; fumaric, 107; malic, 160; and succinic, 374. Thirty compounds which reacted with ninhydrin were found. Nineteen of these were identified as free amino acids and amides. Yields were as follows (μg./gram of spores): aspartic acid, 62; arginine, 195; asparagine, 882; γ-aminobutyric acid, 1335; alanine, 3025; cysteic acid, 278; glycine, 272; glutamic acid, 732; glutamine, 1785; histidine, 147; leucines, 895; lysine, 277; methionine sulfone, 705; proline, 59; phenylalanine, 333; serine, 385; threonine, 452; tyrosine, 378; and valine, 528.

Adenosine di- and tri-phosphates were present in about equal amounts and combined made up 0.23 per cent of the dry weight. Proof of identity of adenosine triphosphate is given and a modified method for isolating adenosine polyphosphates is described in detail. Spores of seven fungi other than *N. sitophila*, including 3 obligate plant parasites, also yielded considerable amounts of adenosine polyphosphates. A glutathione equivalent of 180 μg. of free sulfhydryl groups per gram of spores was found in homogenates.

The data show that *N. sitophila* conidia are similar in composition to spores from other fungi but are considerably higher in ash, protein and nucleic acids than mycelium and vegetative tissues of higher plants.

INTRODUCTION

Studies on the mode of action of fungicides have raised many questions

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[1] This work was supported in part by a grant from the Rockefeller Foundation.

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about the composition of fungus spores and the status of naturally occurring biochemical intermediates, particularly phosphorylated compounds, in the cytoplasm. Although the chemical makeup of spores of a few fungi has been partially elucidated (15, p. 86; 39) practically nothing has been reported previously in this regard about conidia of *Neurospora sitophila* (Mont.) Shear & Dodge. Inasmuch as most of the work on fungicidal action in this laboratory has been done on *N. sitophila* conidia the principal metabolic intermediates and structural components of the spores have been investigated. The results are reported here and compared with data on spores and mycelia of other fungi and cells of other organisms insofar as comparisons are feasible.

MATERIALS AND METHODS

Most of the data are concerned with conidia of *N. sitophila*. However, comparative assays of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) were made on spores of seven additional fungi. The names, sources and spore forms of these are listed below:

Fungus	Source	Spore form
Uromyces phaseoli (Pers.) Wint.	Pinto bean (*Phaseolus vulgaris* L.)	Uredospores
Sphaerotheca pannosa (Wallr.) Lév.	Rose (*Rosa* sp. var. Better Times)	Conidia
Puccinia graminis tritici Eriks. & Henn	Wheat (*Triticium aestivum* L.)	Uredospores
Phytophthora infestans (Mont.) DeBy	Culture	Sporangiospores
Aspergillus niger van Tiegh	Culture	Conidia
Alternaria oleracea Milbraith	Culture	Conidia
Monilinia fructicola (Wint.) Honey	Culture	Conidia

N. sitophila was cultured and conidia were harvested as described previously (27). Spores of obligate parasites were collected from leaf surfaces by the vacuum technique used for harvesting *N. sitophila* spores. *P. infestans* and *A. niger* were cultured on cooked whole wheat grains (50 grams of wheat and 50 ml. of water autoclaved for 20 minutes at 15 p.s.i.) in 250-ml. flasks. The spores were collected by adding water and shaking the flasks. *A. oleracea* and *M. fructicola* were cultured on potato-dextrose agar and the conidia were released into water by scraping the flooded surface with a rubber policeman. All spores were filtered through a double layer of cheesecloth to remove mycelial fragments and washed by repeated suspension and sedimentation in a centrifuge. The water was changed after each centrifugation and washing was continued until the supernatant solution was clear.

After washing, the spores were suspended in water with a mechanical stirrer and the density of the suspension was adjusted to a standard value by use of a Bausch and Lomb Spectronic 20 spectrophotometer to keep the quantity of spores approximately constant from test to test. Dry weights were then determined on 2-ml. aliquots of the suspension after drying the spores in 5-ml. crucibles at 100° C. for 6 hours. All data to follow are based on dry weight.

Subsequent to drying, the spores were ashed in the same crucibles in a muffle furnace at 600° C. The quantity of ash was determined by weighing. The ash was dissolved in H_2SO_4 and the phosphorus content was determined by the method of Bernhart and Wreath (2), modified so that the final volume was 10 ml.

To determine the nature and quantity of spore wall components, conidia were disintegrated quantitatively with a Raytheon 250 W., 10 kc. Sonic Oscillator.[2] The spore walls were separated from cytoplasmic constituents by centrifuging the spore homogenate at about 500×g. for 5 minutes. The walls were washed by centrifuging 5 times with fresh distilled water and twice with 80 per cent ethanol containing 2 per cent formic acid. After washing they were suspended in a known volume of water and two 5-ml. aliquots were dried in weighing bottles at 100° C. for 6 hours and weighed.

A sample of the walls was tested qualitatively for chitin and cellulose by the van Wisselingh procedure (6) and for pectins with ruthenium red (37, p. 60). To determine the amount of chitin present 20 mg. of wall material were transferred in water to hydrolysis tubes and an equal volume of concentrated HCl was added. The tubes were sealed and the contents hydrolyzed for 6 hours in a boiling water bath. After hydrolysis the tubes were opened and the contents dried over KOH in a vacuum desiccator. The glucosamine was then determined quantitatively as described by Boas (4) and by alkaline deamination and determination of ammonia as described by Tracey (44).

Hemicellulose polysaccharides were extracted from the walls and determined quantitatively as described by Jermyn (18). The KOH-insoluble residue after extracting the hemicelluloses was washed thoroughly with water, ethanol and acetone to remove the KOH. It was then air-dried and weighed. The chitin content of this residue was determined as indicated above.

Protein was determined in the wall-free homogenate by weight and by the biuret procedure. For biuret determinations aliquots of the homogenate containing between 5 and 10 mg. of protein were transferred to 15-ml. centrifuge tubes and the protein was precipitated by making the solution 5 per cent with respect to trichloroacetic acid (TCA). The protein was collected by centrifugation and the biuret reaction was carried out as described by Robinson and Hogden (36). Standards were prepared with crystalline pepsin.

For protein weight determinations the homogenate was made 5 per cent with respect to TCA to remove some of the carbohydrate and to precipitate the protein. The precipitate was extracted several times with hot 80 per cent ethanol to remove lipids and then with 10 per cent NaCl at 85° C. for 6 hours to remove nucleic acids (47). The protein was washed with water until all of the NaCl was removed and then with ethanol and finally hot acetone. It was then oven-dried overnight at 100° C. and weighed. Carbohydrate in the final product was determined with anthrone (45) and corrected for in the final value. Nitrogen content was determined by the Kjeldahl procedure (3, p. 11).

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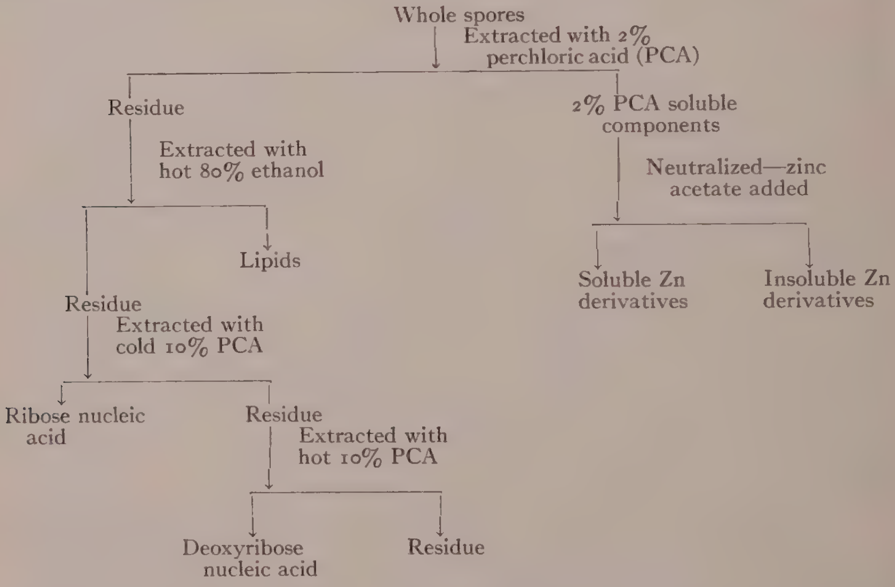
[2] Manufactured by the Raytheon Manufacturing Company, Waltham, Massachusetts.

Inorganic phosphorus was determined by one or both of the methods described by Fiske and Subbarow (14) and Bernhart and Wreath (2). When inorganic phosphate was determined in the presence of ADP, ATP or other labile esters the Bernhart-Wreath method was used to avoid hydrolyzing the esters.

The distribution of phosphorus in the spores was determined by differential extraction of various components according to the following flow sheet:


Whole spores
Extracted with 2% perchloric acid (PCA)
Residue
2% PCA soluble components
Extracted with hot 80% ethanol
Neutralized—zinc acetate added
Lipids
Soluble Zn derivatives
Insoluble Zn derivatives
Residue
Extracted with cold 10% PCA
Ribose nucleic acid
Residue
Extracted with hot 10% PCA
Deoxyribose nucleic acid
Residue

Each of the above fractions was hydrolyzed by heating for 2 hours with H_2SO_4 and hydrogen peroxide and the liberated phosphorus was assayed by the Bernhart-Wreath method.

Adenosine polyphosphates and inorganic phosphate were extracted with 5 per cent TCA or 2 per cent perchloric acid (PCA) in the cold. Five volumes of cold TCA or PCA were added to spore pellets containing about one gram of spores in a series of graduated 15-ml. centrifuge tubes. The spores were suspended in the solvent for 90 minutes at 5° C., after which they were centrifuged. The extract was decanted, neutralized with concentrated NaOH and frozen. This procedure was repeated 4 times and the extracts were combined. An excess of 25 per cent zinc acetate solution was added and the extract was frozen. The precipitate was collected by centrifugation as the ice melted. It was resuspended in 10 volumes of cold ethanol and resedimented to remove most of the residual TCA or PCA. It was then taken up in 100 volumes of 1*N* NH_4OH and the solution was passed through an 18×30 mm. column of chloride form Dowex 1-X10 resin, 200 to 400 mesh. The column was prepared in 18×200 mm. glass tubes drawn to a 4- to 5-mm. opening at the bottom. A glass wool plug was placed at the bottom to hold back the resin. After sorption, four bed volumes of 1*N* NH_4OH were run through the column to remove the zinc. This was followed by distilled water until the effluent liquid was neutral to litmus paper.

Adenosine monophosphate was eluted with an aqueous solution of 0.003*N* HCl, ADP with a mixture of 0.01*N* HCl and 0.02*M* NaCl, and ATP with 0.01*N* HCl and 0.2*M* NaCl as described by Cohn and Carter (10). In each case elution was continued until the ultraviolet absorption of the effluent solution at a wavelength of 260 mμ became negligible.

The amounts of ADP and ATP were determined at 260 mμ in a Beckman Model DU spectrophotometer using a path of 1 cm. Standards were prepared with chromatographically pure ADP and ATP in the appropriate solvent.

In determining the composition of conidial ATP, eluates from the column were adjusted to pH 8.0 with concentrated NaOH and made 50 per cent with respect to ethanol. ATP was then precipitated by adding a few ml. of 25 per cent barium acetate solution. The solution was cooled to 0° C. and the barium ATP was collected by centrifugation.

The labile phosphate groups of ATP were hydrolyzed in 1*N* HCl held at 100° C. for 7 minutes (46). The liberated phosphorus was determined by the Bernhart-Wreath method. The pentose content was determined with orcinol according to the method of Mejbaum (21). The hexokinase reaction was carried out in Warburg respirometers at 30° C. using the system described by Colowick and Kalckar (11). Paper chromatography was done on Whatman No. 1 paper using the iso-amyl alcohol-phosphate solvent described by Cohn and Carter (10). After drying the papers the spots were located with an ultraviolet lamp.

For the hexokinase reaction and for paper chromatography it was necessary to convert the barium salt of ATP to the sodium salt. This was done by dissolving the barium salt in about 2 ml. of cold 2*N* HCl, adding a slight excess of Na_2SO_4 and then neutralizing the solution with NaOH. The $BaSO_4$ was removed by centrifugation.

Lipids were extracted in two ways, depending on subsequent tests to be made. When nucleic acids only were to be determined, the lipids were extracted with hot 80 per cent ethanol. When analysis of the lipids *per se* was made, the spores were extracted 5 times at room temperature with about 10 volumes of a mixture of 80 per cent ethanol and 2 per cent formic acid, which also extracted sugars, amino acids, etc. The extracts were combined and the liquid evaporated under partial vacuum. After drying, the residue was extracted 4 times with 25-ml. portions of petroleum ether. The petroleum ether extract was passed through a 1 × 7 cm. silica gel (100 mesh) column which retained the lipids. The lipids were then separated into 6 categories by differential elution as described by Fillerup and Mead (13). Each fraction was dried at 100° C. for 6 hours and weighed.

After extracting the lipids the remaining residue from the original ethanol-formic acid extract was taken up in 80 per cent ethanol and passed through a 1 × 5 cm. column of hydrogen form Dowex 50-X8 resin, 200 to 400 mesh, to remove the amino acids, which were subsequently eluted according to the method of Plaisted (32). The amino acid solution was taken to dryness in a stream of air under partial vacuum. The residue was dissolved in 0.5 ml. of 50 per cent ethanol, spotted on Whatman No. 3 paper, chromatographed and determined quantitatively as described by Porter *et al.* (33).

The effluent 80 per cent ethanol solution from the Dowex 50 column after removal of amino acids was adjusted to pH 8.0 with NaOH and passed through a 1 × 10 cm. column of borate form Dowex 1-X10 resin, 200 to 400 mesh, which retained the sugars and phosphorylated compounds. These were then separated by gradient elution with sodium borate and sodium chloride as described by Parr (30) and then determined quantitatively with anthrone (45) after evaporating the water in a current of air. Supplementary evidence of identification was obtained by paper chromatography (31). After development the dried chromatograms were sprayed with naphthoresorcinal-phosphate reagent (5) to locate the spots. Phosphorylated compounds were chromatographed and located as described by Mortimer (24).

Nucleic acids were extracted with 10 per cent PCA as described by Ogur and Rosen (26) after removal of lipids. Deoxyribose nucleic acid (DNA) was extracted after removal of RNA as described by the same authors. The amounts of nucleic acids in the extracts were determined by ultraviolet absorption at 260 mμ (26) in a Beckman Model DU spectrophotometer with a path of 1 cm. and from phosphorus values after digestion of the acids with H_2SO_4 and hydrogen peroxide. Standards were prepared with commercial yeast RNA and sperm DNA. Supplementary evidence of identification was obtained by the diphenylamine reaction for DNA (12) and the orcinol reaction for RNA (21).

Keto acids were extracted with $2N$ HCl containing 100 μg. of 2,4-dinitrophenylhydrazine per ml. A single overnight HCl extraction at 5° C. was made. The spores were filtered on a Büchner funnel and extracted further with a volume of ethyl acetate equal to that of the filtrate. The ethyl acetate and HCl extracts were transferred to a separatory funnel and shaken together for about 5 minutes. After separation, the water phase was drawn off and shaken again with fresh ethyl acetate. This was repeated until all of the colored material was in ethyl acetate. The ethyl acetate was then extracted by shaking with successive 10-ml. portions of 10 per cent sodium carbonate solution until no further color appeared in the carbonate solution. The carbonate extracts were combined and acidified with concentrated HCl. This was extracted with ethyl ether until all of the yellow compounds were in the ether phase. The ether was then allowed to evaporate and the 2,4-dinitrophenylhydrazones were taken up in a known volume of ethanol suitable for spotting chromatograms.

Chromatography was carried out on Whatman No. 1 paper in the solvent system described by Cavallini and Frontali (7), with addition of 1 per cent ammonium hydroxide. After development for 20 hours ascending, the chromatograms were dried and the spots were cut into small squares. The 2,4-dinitrophenylhydrazones were eluted from the paper in test tubes with 50 per cent aqueous ethanol for about one hour and the amounts determined with a Beckman spectrophotometer at a wavelength of 380 mμ. Standards for chromatography and spectrophotometry were prepared with purified 2,4-dinitrophenylhydrazones synthesized from known keto acids.

Nonketo, nonvolatile acids were extracted from the spores with $6N$ aqueous formic acid. Five extractions were made, the extracts were combined and the solvent was removed in a stream of air under partial vacuum. The residue was taken up in water and applied to a 1×15 cm. column of formate form Dowex 1-X10 resin, 200 to 400 mesh. After sorption, the acids were eluted and the amounts determined as described by Palmer (29). Supplementary evidence of identification was obtained by paper chromatography, using solvent F described by Stark, Goodban and Owens (41).

Total sulfhydryl content of spore homogenates prepared by sonic treatment was determined by the modified nitroprusside method described by Grunert and Phillips (17).

Total free carbohydrate was extracted with boiling water and determined with anthrone (46) using glucose as the standard. All carbohydrate values to follow are glucose equivalents.

RESULTS

Water. The water content of the spores as they were harvested from cultures was found to vary widely with the age and extent of desiccation of the cultures. Much more reproducible values were obtained by drying the spores in air or over $CaCl_2$. Air-dried they contained an average of 9.1 per cent water (Table I). Dried over $CaCl_2$ for 48 hours they contained 5.1 per cent moisture which was probably roughly equivalent to bound water.

Ash. The residue after combustion of the spores at 600° C. averaged 6.8 per cent of the dry weight (Table I). Phosphorus in the ash amounted to 23 per cent. However, when phosphorus was determined after wet ashing an equivalent amount of spores with H_2SO_4 and hydrogen peroxide, it amounted to 28 per cent of the ash. This indicates that phosphorus amounting to 5 per cent of the ash was lost during combustion, probably due to incomplete binding of the volatile P_2O_5 in the ash. Other components of the ash were not determined.

Walls. The proportion of the spores made up by walls and various wall

TABLE I

SUMMARY OF MAJOR COMPONENTS OF NEUROSPORA SITOPHILA CONIDIA

Component	Mg./gram of spores	Summary percentage of dry weight
Water (air-dried)	91	
(dried over $CaCl_2$)	51	
Ash	68	6.8
Phosphorus	19.1	
Organically bound	16.8	
Inorganic	2.3	
Spore wall	272	27.2
Polysaccharides (hemicelluloses)	144	
Chitin	15	
Cellulose, etc.	85	
Other (H_2O, losses, etc.)	28	
Protein (biuret)	252	
(weight)	258	25.8
Lipids	177	17.7
Nucleic acids	104	
RNA (P value)	90	9.0
(absorption)	83	
DNA (P value)	14	1.4
(absorption)	8	
Carbohydrates (hot water soluble glucose equivalent)	93	9.3
Free sugars (glucose)	0.60	
Sugar phosphates	1.04	
Hexose-phosphate	1.00	
Hexose-diphosphate	0.04	
Triose phosphate	0.04	
Organic acids	1.95	0.2
Keto	0.08	
Nonketo	1.87	
Free amino acids and amides	12.73	1.3
Adenosine polyphosphates	2.35	
ATP	1.18	
ADP	1.17	
Total acidity (milliequivalents)	23.04	
Total free SH groups (glutathione equivalents)	0.18	
Total	98.67*	98.7

* Oven-dried spores—water excluded.

components is shown in Table I. Chitin accounted for 15 per cent of the 24 per cent KOH-insoluble portion of the walls, the remainder being polysaccharides. KOH-insoluble material comprised 32 per cent of the wall solids and was made up partially of cellulose. It was not established whether lignin or other KOH-insoluble material was present.

Over 50 per cent of the wall was made up of polysaccharides (hemicelluloses) soluble in 24 per cent KOH. These precipitated upon acidifying the extract and gave a strong blue color with iodine. A small amount of the air-dried wall was not recovered either in the KOH-insoluble fraction or by alcohol precipitation of material in the KOH-soluble fraction. Part of the loss may have been due to water since the material was dried only in the air at room temperature, but tests with iodine indicated that a small amount of alcohol-soluble material was present in the filtrate after removal of the alcohol-insoluble polysaccharides. Whether this was derived by degradation from long-chained polysaccharides during KOH extraction or was present in the original wall is unknown. Losses also occur due to destruction of glucosamine during acid hydrolysis of the walls. According to Tracey (43) these might amount to as much as 25 per cent of the glucosamine, which in the present instance would be between 1 and 2 per cent of the total wall material.

Protein. Data in Table I show that the protein content of the spores was 25.2 per cent based on biuret determinations and 25.8 per cent based on weight corrected for moisture and about 1 per cent residual carbohydrate. The protein contained 15.4 per cent nitrogen, which is close to the theoretical 16.0 per cent for simple proteins. The slightly low value may have been due in part to conjugation, since the isolated protein had a pink color indicating the presence of an unknown chromaphore. The chromaphore was released upon acid hydrolysis.

Nucleic acids. Nucleic acids were determined from phosphorus values and by ultraviolet absorption. It can be seen from data in Table I that values for DNA obtained by the two methods did not agree. Disagreement was found to be related, in part at least, to the fact that the normal absorption maximum was shifted from near 260 mμ for purified DNA to about 275 mμ for spore extracts. In view of this, phosphorus would appear to be the better of the two measurements. On the basis of phosphorus content, RNA was found to make up 9.0 per cent and DNA 1.4 per cent of the spore weight. These values were calculated on the basis of RNA and DNA standards with approximately 9 per cent phosphorus. The ratio of RNA to DNA averaged 6.4.

The orcinol reaction was positive for RNA extracts and negative for DNA extracts (Table II). The opposite was true for the diphenylamine reaction, indicating an insignificant amount of cross-contamination between the two fractions.

TABLE II

ANALYSIS OF NUCLEIC ACID FRACTIONS FROM NEUROSPORA SITOPHILA CONIDIA

Sample	Orcinol reaction	Diphenylamine reaction	Relative ultraviolet absorption*
DNA standard	−	+	1.00
Conidial DNA fraction	−	+	0.57
RNA standard	+	−	1.05
Conidial RNA fraction	+	−	0.92

* Based on phosphorus contents equivalent to the DNA standard.

Carbohydrates. Hot water-soluble carbohydrates accounted for 9.3 per cent of the spore solids (Table I). Although the material reacted with anthrone it did not produce a blue color with iodine and therefore was not starch. Less than 5 per cent of the total passed through chromatographic columns under the conditions employed for separating free sugars and phosphorylated intermediates. Thus the bulk of the carbohydrates are unidentified but resemble glycogen in some respects.

The small portion of carbohydrate separated on chromatographic columns was found to be made up principally of free glucose, hexose-phosphate, hexose-diphosphate, 3-phosphoglyceric acid and an unknown component giving high background color with anthrone in the first 50 samples (Table I). Several small peaks occurred in the profile of the elution curve preceding glucose. These indicate that a number of other free sugars were present in very small amounts. Further study on these is in progress.

Lipids. Lipids, which made up 17.7 per cent of the spore weight (Table I), were separated into six groups on a silica gel column. The amount of lipid in each group is shown in Table III. The nature of the phospholipids, the predominant lipids, is now being studied but comprehensive data are not yet available.

Phosphorus. The spores contained 1.9 per cent phosphorus (Table I) as

TABLE III

CONTENT OF VARIOUS GROUPS OF LIPIDS IN NEUROSPORA SITOPHILA CONIDIA

Eluting agent	Lipid	Mg./gram of spores
1% Ethyl ether in petroleum ether	Sterol esters	1.8
4% Ethyl ether in petroleum ether	Triglycerides	1.1
10% Ethyl ether in petroleum ether	Sterols	3.5
50% Ethyl ether in petroleum ether	Fatty acids	2.0
50% Ethyl ether, 50% methanol, 1% HCl (1st fraction)	Carotenoids	30.1
50% Ethyl ether, 50% methanol, 1% HCl (2nd fraction)	Phospholipids	138.5
Total		177.0

determined after digestion of the spores with H_2SO_4 and hydrogen peroxide. This was distributed among all major fractions as shown in Table IV. Proximate calculations of the lipid fraction as lecithin gives about 18 per cent lecithin equivalent based on phosphorus content. This value is close to the 17.7 per cent lipid found by weight and agrees with the fact that the bulk of the lipids was eluted from silica gel columns with the phospholipid solvent.

The principal phosphorus compounds soluble in 2 per cent PCA were found to be inorganic phosphorus, hexose-phosphate, hexose-diphosphate, 3-phosphoglyceric acid, ATP and ADP. These, however, did not account for all of the 2 per cent PCA-soluble phosphorus. The presence of other phosphorus-containing compounds was indicated also by unpublished data on metabolic interconversions requiring other phosphorylated intermediates and phosphorus-containing coenzymes.

Adenosine polyphosphates. The high energy adenosine polyphosphates were of particular interest because of their relation to synthetic processes. They comprised a little more than 0.2 per cent of the dry weight of the spores (Table I). ADP and ATP were present in about equal quantities on an average although in 6 tests on different lots of spores the ratio of ATP to ADP ranged from 0.7 to 1.3.

ATP was isolated at first by the method used for animal tissues (46, p. 160). In this procedure the phosphates are precipitated as barium salts. It was found later, however, that there was an important discrepancy between the amount of adenine-containing compounds in the precipitate and the amount recovered from chromatographic columns. The method was then modified by substituting zinc for barium as the precipitating

TABLE IV

DISTRIBUTION OF PHOSPHORUS AMONG VARIOUS FRACTIONS OF NEUROSPORA SITOPHILA CONIDIA*

Fraction	Phosphorus, mg.	Percentage of total phosphorus
Whole spores	90.65	100.0
2% Perchloric acid extract	25.20	27.8
Insoluble zinc derivatives	15.70	
Inorganic P	10.65	
Soluble zinc derivatives	9.50	
Petroleum ether extract (lipids)†	10.10	11.1
Cold 10% perchloric acid extract (RNA)	46.60	51.4
Hot 10% perchloric acid extract (DNA)	7.40	8.2
Spore residue	3.50	3.9
Total	92.80	102.4

* Samples averaged 4.74 g. of spores (dry wt.).

† The spores were extracted with 80% alcohol—2% formic acid. The extract was taken to dryness and the residue extracted with petroleum ether.

agent and considerably better yields were obtained (28). The zinc salts had the added advantage that they could be dissolved in 1N NH_4OH and applied to the Dowex 1 column directly without first removing the cation as is necessary in the barium precipitation procedure.

ATP and ADP from conidia were found to exhibit generally the same chromatographic properties as ATP and ADP from other sources since they separated on Dowex 1 chromatographic columns under the same conditions. They moved on Whatman No. 1 paper in Cohn and Carter's solvents to Rfs averaging 0.93 for ATP and 0.85 for ADP, which were almost identical to Rf values obtained for authentic ATP and ADP on the same papers. Cochromatographed, the authentic and test compounds did not separate.

Absorption characteristics of conidial ATP also were almost identical to those of authentic ATP (Fig. 1). There was slight skewing of the curve in case of the conidial preparation in the region between 275 and 300 mμ, suggesting the presence of contaminating material that absorbed in this region.

Conidial ATP gave essentially the theoretical ratio of adenine, pentose and phosphorus and yielded the expected 2 moles of phosphorus per mole of ATP upon hydrolysis in 1N HCl at 100° C. for 7 minutes (Table V). It was active as a phosphate donor in the hexokinase phosphorylation of

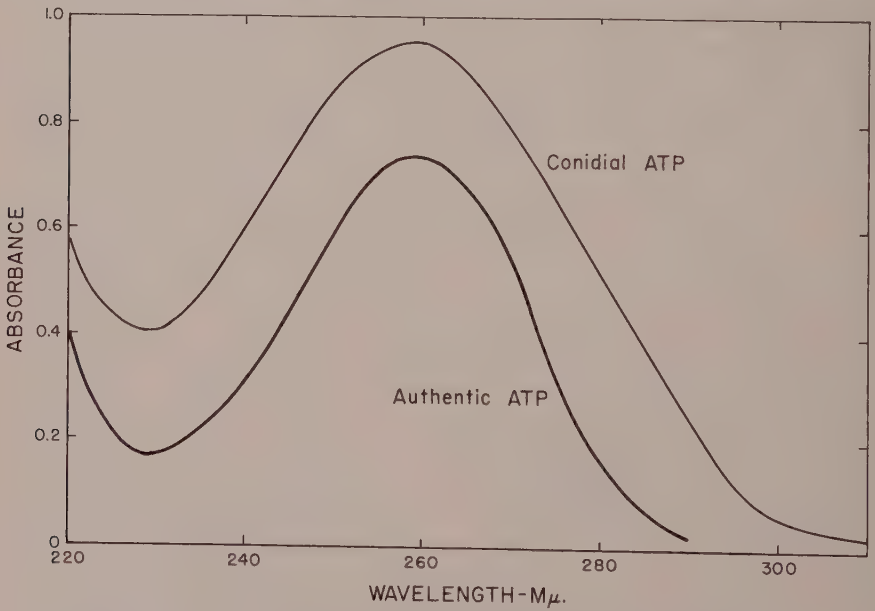


FIGURE 1. Ultraviolet absorption curves for authentic ATP and for ATP from *Neurospora sitophila* conidia. Solvent: 0.01N HCl and 0.2M NaCl in water.

TABLE V

COMPOSITION OF ADENOSINE TRIPHOSPHATE FROM NEUROSPORA SITOPHILA CONIDIA*

Component	Found, µg.	Theory, µg.	Difference, µg.
Pentose	87.0	85.5	1.5
Labile P†	35.6	35.3	0.3
Total P	57.6	52.8	4.8

* Samples average 289 µg. of ATP (free, anhydrous acid) based on ultraviolet absorption.

† Hydrolyzed in 7 min. in 1*N* HCl at 100° C.

glucose. Under the conditions described by Colowick and Kalckar (11), 3 mg. of conidial ATP, based on U. V. absorption, gave an initial rate of CO_2 release from carbonate of 82 µl./hr. Authentic, chromatographically pure ATP, with equivalent ultraviolet absorption, released 126 µl. of CO_2 per hour, which indicated that about 35 per cent of the conidial preparation was inactive.

After identity of ATP from *N. sitophila* conidia was established, a survey was made of the occurrence of ATP and ADP in spores of seven additional fungi. Spores of all fungi tested yielded substantial amounts of both compounds (Table VI).

Organic acids. The total acidity of the spores was determined by titration of wall-free homogenates with NaOH against the glass electrode of a Beckman pH meter. The acid equivalent is given in Table I. This, however, includes all groups capable of combining with cations and does not represent the amount of organic acids present. The principal nonvolatile organic acids were subsequently separated and measured individually. The total amount of the acids identified is given in Table I and the amounts of each acid are shown in Table VII.

Although volatile acids were not determined it is apparent from the acid equivalent value of the whole cytoplasm that acids did not make up a large part of the cytoplasmic contents. Free high molecular weight fatty

TABLE VI

ADENOSINE DI- AND TRI-PHOSPHATE CONTENT OF SPORES FROM VARIOUS FUNGI

Spores of	ATP, mg./g.	ADP, mg./g.	Total ATP+ADP, mg./g.
Uromyces phaseoli	0.190	0.200	0.390
Sphaerotheca pannosa	0.980	0.900	1.880
Puccinia graminis tritici	0.290	0.850	1.140
Aspergillus niger	0.405	0.860	1.265
Monilinia fructicola	5.370	6.900	12.270
Phytophthora infestans	5.200	6.200	11.400
Alternaria oleracea	1.520	2.000	3.520

TABLE VII

NONVOLATILE ORGANIC ACID CONTENT OF NEUROSPORA SITOPHILA CONIDIA

Acid	μG./gram of spores
Keto acids*	
Glyoxylic	6 (28)
α-Ketoglutaric	51 (139)
Pyruvic	24 (78)
Oxalosuccinic	Trace
Nonketo acids	
Succinic	374
Malic	160
Citric	1232
Fumaric	107

* Expressed as μg. free keto acid per gram of spores. The corresponding values for the 2,4-dinitrophenylhydrazones are given in parentheses. The latter are converted to free acid values by the conversion factor $(1-\frac{200}{x-16})y$, where x is the molecular weight and y the value found for each 2,4-dinitrophenylhydrazone.

acids occurred only in very small amounts as was indicated by lipid fractionations (Table III).

Free amino acids and amides. The total free amino acid and amide content is given in Table I and the individual compounds are given in Table VIII. Thirty compounds reacting with ninhydrin were found. Of these 11

TABLE VIII

FREE AMINO ACIDS AND AMIDES FROM CONIDIA OF NEUROSPORA SITOPHILA*

Amino acid or amide	μG./gram of spores
Aspartic acid	62
Arginine	195
Asparagine	882
γ-Aminobutyric acid	1335
Alanine	3025
Cysteic acid	278
Glycine	272
Glutamic acid	732
Glutamine	1785
Histidine	147
Leucines	895
Lysine	277
Methionine sulfone	705
Proline	59
Phenylalanine	333
Serine	385
Threonine	452
Tyrosine	378
Valine	528
Total	12725

* Eleven additional compounds that reacted with ninhydrin were found but have not yet been identified.

are unidentified. Alanine was the dominant amino acid and glutamine the dominant amide. Studies to determine the identities of the unknowns are in progress. Both methionine sulfone and cysteic acid are probably derivatives formed during chromatography and the values given do not represent the amounts of these compounds *per se* in the spores. The precursor of cysteic acid has not been established but it is reasonably certain that methionine sulfone was derived from methionine.

Free sulfhydryl groups. Free sulfhydryl groups, calculated as glutathione, were present in small quantities, *ca.* 180 μg./gram of spores (Table I).

DISCUSSION

N. sitophila conidia were found to be similar in many respects to spores of other filamentous fungi. The ash content is somewhat higher than that generally found for mycelium but similar to that reported by Rennerfelt (35) for *A. niger* spores and by Sumi (42) for *A. oryzae* spores. Ash content of higher plants is difficult to compare with that of the spores since it varies from 1 to 15 per cent (22, p. 319) depending on the species and plant part examined.

The major portion of mycelial walls of most fungi is made up of polysaccharides (15, p. 76) which is similar to the situation found for walls of *N. sitophila* spores. The polysaccharides are believed to be infiltrated among a framework of cellulose, callose, lignin and chitin (15, p. 97). The presence of cellulose and chitin in the present tests suggests that the general framework of spore walls is similar to that suggested for mycelial walls. Chitin usually makes up a relatively small portion of the total wall but its occurrence in many fungi (15, p. 90) and in certain algae belonging to the Chlorophyceae (43) is well established and seems to be a characteristic of certain related lower organisms. It does not occur in bacteria and its presence in yeast is controversial (43).

Foster (15, p. 103) states that there are no precise data available on protein content of filamentous fungi, probably owing to difficulties in extracting the protein quantitatively. This difficulty was eliminated in the present study by sonic disintegration of the spores, which released the cytoplasm quantitatively. It was thus possible to isolate the proteins and take weight, biuret, and nitrogen measurements directly instead of relying on alcohol-insoluble nitrogen values, which obviously are generally too high because of the presence of nucleic acids and other nitrogenous material.

The protein value obtained for *N. sitophila* spores, i.e., about 250 mg./gram, was similar to that reported for uredospores of *P. graminis tritici* by Shu *et al.* (39) who disintegrated the spores in a ball mill. Crude values based on alcohol-insoluble nitrogen are available for the mycelium of several fungi also. Pruess *et al.* (34) give a range of 13.7 to 43.7 per cent with an average of 31.6 per cent for mycelium of 24 species of Aspergilli and Penicillia. Block and Weiss (3, p. 326) give an average nitrogen value equivalent to about 42 per cent protein for several fungi. In order to compare values with those for *N. sitophila* conidia it is necessary to include nucleic acid and chitin values for the spores. When this is done the combined nitrogenous components make up slightly more than 40 per cent of the spore weight.

Higher plant protein, based on nitrogen, ranges from about 5 per cent for leafy portions to about 25 per cent for the average of legume seeds (3, p. 316).

In the present tests it is recognized that errors were inherent in nucleic acid measurements whether the acids were determined by ultraviolet absorption or on the basis of phosphorus content. In the first instance interfering material caused the absorption maximum to shift. Phosphorus values, on the other hand, are probably slightly too high because of concomitant extraction of nonnucleic acid phosphorus with PCA, especially in the DNA fraction. However, despite this, phosphorus values appear to be more realistic than absorption values.

Belozersky (1) states that mold mycelium usually contains 3 to 6 per cent nucleic acids whereas in bacteria the nucleic acid content ranges from 10 to 28 per cent. The nucleic acid content of the spores is thus somewhat higher than for mycelium but lower than for bacteria. The RNA/DNA ratio is intermediate between the ratio of 3.4 for *Escherichia coli* and 11 for baker's yeast reported by Mitchell and Moyle (23).

Higher plant vegetative tissues generally have a lower nucleic acid content than that found for spores. According to Fries (16) nucleic acid phosphorus of leaves of a variety of deciduous trees varies between 0.12 and 0.78 mg./g. dry weight compared to 1.15 mg./g. of spores.

The lipid content of fungi varies widely with species, strain, and composition of the culture medium (15, p. 114). In mycelium of certain fungi oils and free fatty acids accumulate. This is different from the situation in *N. sitophila* conidia where the bulk of the lipids are phosphorus-containing compounds as shown both by elution characteristics on silica gel columns and by phosphorus content of the lipid fraction. However, the proportion of lipid is similar to the 19.7 per cent found by Shu *et al.* (39) for uredospores of *P. graminis tritici.*

Phosphorus distribution among the various cell components has not been determined for spores or mycelium of other fungi. Qualitatively, however, the distribution of phosphorus and the presence of phosphorylated intermediates is in agreement with the findings of Mann (20) and Semeniuk (38). The presence of phosphorylated intermediates and the high energy phosphates ATP and ADP, furthermore, is in accord with pathways of carbohydrate dissimilation recently elucidated for fungi (8, 9) and would suggest that Nord's view (25) that phosphorylation may not be involved in glucose metabolism is untenable.

It is difficult to compare values for organic acid and amino acid contents of the spores with those for other fungi or other organisms because both are dependent in large part on age and cultural conditions. In other studies with *N. sitophila* spores, organic acids and amino acids were found to be utilized as endogenous substrates in respiration. The quantitative values reported here, therefore, must be looked upon as approximate, suggesting an order of magnitude rather than absolute amounts, since they can be expected to change if cultural conditions are changed or if spores of different age are assayed.

A similar study of organic acids in uredospores of *Puccinia rubigo-vera tritici* and *P. graminis tritici* was made by Staples (4) who was able to demonstrate the presence of malonic, citric, succinic, malic, fumaric and aconitic acids. No indications of malonic or aconitic acids were found in *N. sitophila* spores, although it is possible that traces too small to be apparent by the technique used were present. Comparable studies with spores of other fungi apparently have not been made. However, a great many organic acids are known to be synthesized by fungi and in some cases fungi are used for commercial production of specific acids. Foster (15, p. 351) has reviewed these aspects of the fungi in some detail and should be consulted if further comparisons are desired.

Qualitatively the organic acids present in *N. sitophila* conidia are in accord with the operation of Krebs cycle, as was suggested previously (27). The presence of glyoxylic acid suggests further that the modified cycle as described by Kornberg and Krebs (19) might also operate. The failure to demonstrate the presence of all Krebs cycle intermediates would not rule out the cycle as a functioning pathway in acid metabolism since the state of equilibrium between the various acids is unknown.

Reports on the free amino acids and free sugars in fungi appear to be lacking. Most of the available data deal with hydrolyzates which are not comparable to the present values for free amino acids or sugars.

Although more than 98 per cent of the cell solids have been accounted for in the various fractions assayed, it is obvious that only major classes of compounds have been determined and that within each class there may be a large number of compounds present in little more than trace amounts. The present report does not intend to discount these or to ignore their importance in metabolism and growth.

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Note

GROWTH RESPONSES OF A CYPHOMANDRA-LYCOPERSICON GRAFT

LAWRENCE J. KING[1]

A scion of the common tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) was successfully grafted upon the woody, solanaceous *Cyphomandra betacea* Sendt. While only one such graft was obtained, the responses were so striking that certain of these warrant description at this time. *Cyphomandra betacea* (5), known as the tree tomato, is a native of Peru (4). It is cultivated in New Zealand (2) and other parts of the world. Both the cytological and plant disease relationships have been studied (3, 7). Following the work described here it was found that Walker (6) had made similar grafts but used scions of *Lycopersicon pimpinellifolium* Mill.

Seeds of *Cyphomandra betacea* Sendt. var. Golden Gem were obtained from a commercial seed house (Glecklers' Seedsmen, Metamora, Ohio). The seeds were germinated in the greenhouse in early April, 1952, and then were transplanted to four-inch pots. When the seedlings were about one month old, scions of tomato were inserted by means of a cleft graft prepared from a three-inch stump. The scions consisted of the apical two inches of the primary axis, and were taken from plants grown from seed under the usual conditions prevailing in the greenhouse. The grafted plants were placed in a moist chamber for several weeks.

One grafted plant survived out of some six that were prepared, and when the union appeared firm, the plant was removed from the chamber and placed in the greenhouse for several weeks. A tomato plant of the same variety on its own roots was maintained for general growth observations. In the early part of June the single grafted and nongrafted plants were planted in the field. New shoots grew rapidly from the stock at all times and were difficult to keep under control. Hence a certain amount of stock foliage was permitted to grow throughout the course of these studies.

The grafted tomato plant grew very slowly. During the entire summer in the field (from June to October) the scion attained a length of about 12 inches. The plant on its own roots grew into a normal, healthy, and fruiting plant about two feet in height. Due to a light frost one night late in October the scion was damaged. The grafted plant only was then dug up and removed to the greenhouse where it was grown in a four-inch clay pot during the winter of 1952–1953.

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[1] Senior Research Fellow in Plant Physiology, Union Carbide Chemicals Co. Fellowship, Boyce Thompson Institute for Plant Research, Inc.

Following the death of the scion it was cut back to within four or five inches of the graft union resulting in the production of a new shoot (the callus shoot) from the graft callus. The callus cells appeared to have developed entirely from tomato tissue. The remaining portion of the original scion eventually died, leaving the stump shown in Figure 1 A. This new callus shoot, too, grew very slowly during the winter. During the early spring of 1953 the grafted plant was moved to a six-inch clay pot. At this time the new callus shoot had attained a height of about 12 inches; but with re-potting growth then became much more rapid.

In approximately one year from cutting back the original scion, the newly formed callus shoot attained a height of about 30 inches. In spite of the over-all age of the plant and the poor growth conditions which undoubtedly had their effects, certain physiological responses of the callus shoot seem worthy of note. Perhaps the most striking responses were the root initials that partially emerged along nearly two-thirds of the length of the callus shoot. They never attained a length much greater than 1 to 2 mm. (Fig. 1 A). Epinasty of leaves, the downward curling of leaflets, and swollen nodes represented other physiological responses to this type of graft (Fig. 1 B).

Fruit set and development were poor. During the winter months the early flower clusters abscised. In the spring period the later-formed clusters set fruit which remained dwarfed and green over a period of many months. In fact these fruits never grew any larger than shown (Fig. 1 C), and of course they were many months older than the mature fruit shown in Figure 1 D. These dwarfed fruits were located in inflorescences developing from the primary axis of the callus shoot. It is not known whether this fruit dwarfing is the result of the grafting or of other conditions—possibly environmental. A lateral branch that had developed near the top of the callus shoot produced a cluster of flowers, the majority of which developed into ripened fruit of normal shape and color although small in size. It would appear that fruits formed in closer proximity to the graft callus and the stock foliage were more likely to be dwarfed; while those fruits produced several feet above on the callus shoot appeared to develop more normally. Again environmental factors may be operative here.

Very near the top of the primary axis of the callus shoot, in the last inflorescence produced at this date, a single but an odd-shaped fruit developed. Unlike all the other fruit that had been noted on this plant this particular one was less round in shape and pointed at the stylar end (Fig. 1 D right). Upon sectioning, the green fruit of the former was found to be tri-carpellate. For comparison of shapes a ripe fruit of a greenhouse-grown Bonny Best tomato is shown in Figure 1 D (left), and a green fruit of the

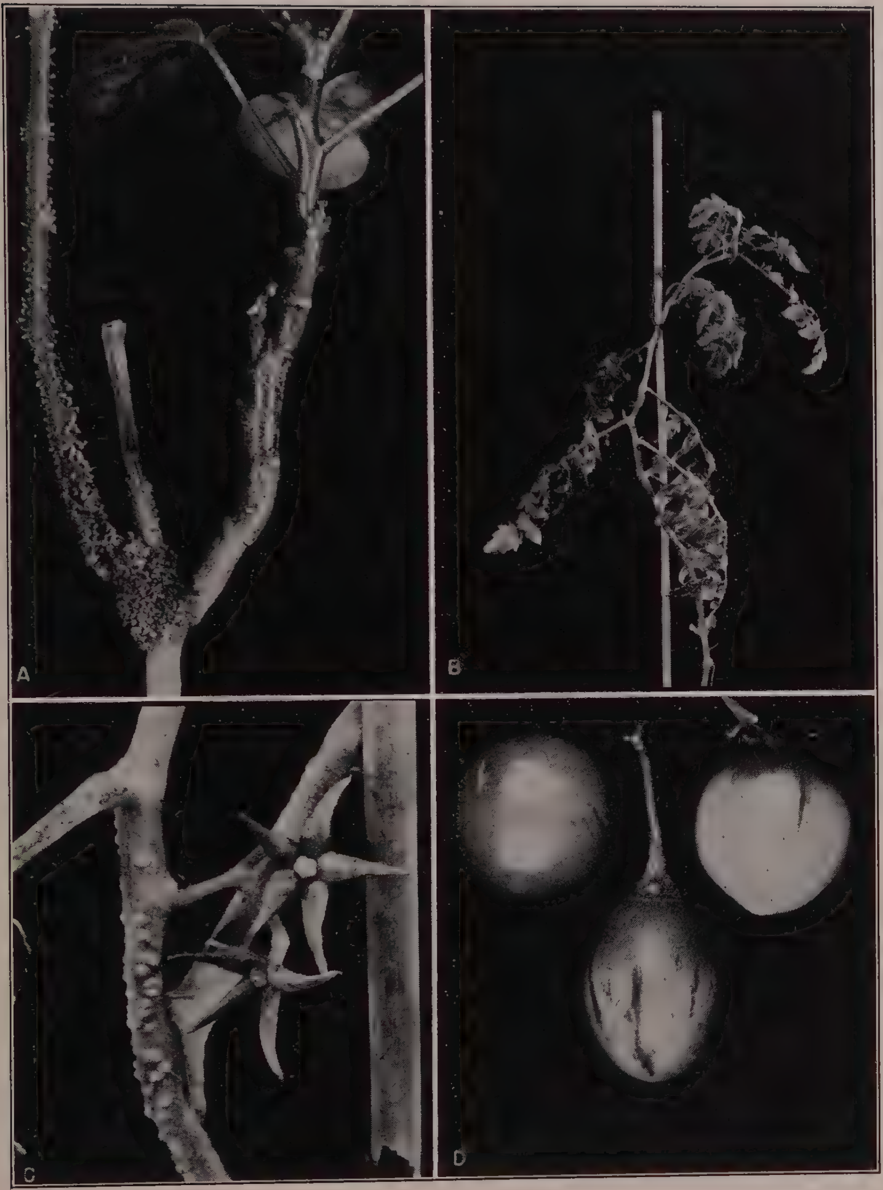

FIGURE 1. Aspects of the *Cyphomandra-Lycopersicon* graft. (A) Left, base of tomato callus-shoot showing root initials; middle, stump of original scion; right, *Cyphomandra* stock showing side branch with foliage. (B) Upper portion of the tomato callus-shoot showing epinastic-like aspect of the leaves. (C) Tomato inflorescence from the callus-shoot showing two dwarfed and undeveloped fruits. (D) Upper left, normal Bonny Best tomato fruit; center, fruit of *Cyphomandra betacea;* right, tomato fruit from the upper portion of the callus shoot.

tree tomato (Fig. 1 D center). It is of interest to note here that Daniel (1) reported obtaining modified fruit shapes in intervarietal pepper grafts.

Grafting procedures such as the present one should be useful in the study of stock-scion physiology, and particularly so because of the known sensitivity of the tomato plant to a diversity of growth-regulating substances. The role of growth substances produced in the foliage of the stock on the growth of the scion has rarely been observed or assessed since it is considered good horticultural practice to remove all such growth. The physiology of dwarfing, and indeed of flowering might well be examined through the use of such intergeneric grafts—exploring the growth of callus shoots, and also varying the amount of stock-foliage growth permitted.

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THE SULFUR METABOLISM OF INSECTS. III. THE METABOLISM OF CYSTINE, METHIONINE, TAURINE, AND SULFATE BY THE HOUSE FLY, MUSCA DOMESTICA L.[1]

Val F. Cotty,[2] S. Mark Henry, and John D. Hilchey[3]

SUMMARY

House flies were either fed or injected with S^{35}-labeled cystine, cysteine, methionine, taurine or sulfate. Extracts, hydrolyzates, and feces were then analyzed by chromatography and autoradiography for radioactive metabolites. Cysteinesulfinic acid, a key intermediate in sulfate formation, appeared on chromatograms of flies injected with cysteine-S^{35}. There appeared to be two pathways for the synthesis of taurine. Taurine seemed to arise from cystine via hypotaurine. When methionine was the precursor, hypotaurine was not found in detectable quantities but there was an increase in cysteic acid, a recognized intermediate in taurine synthesis. In the latter case relatively more taurine-S^{35} was formed than when cystine-S^{35} was administered. Sulfate-S^{35} was not incorporated into soluble organic compounds. Taurine-S^{35} gave rise to three unidentified metabolites, one of which may be isethionic acid. The results indicate that house flies possess an active transulfuration system.

INTRODUCTION

Hilchey *et al.* (15) initiated investigations of sulfur metabolism in house flies with a study of the conversion of injected cystine-S^{35} into taurine and sulfate. There were indications that metabolites of cystine other than taurine and sulfate were present but methods had not been developed for their detection and identification. The house fly apparently resembled several other insect species in some of its transulfuration mechanisms but was unlike the cockroach (14), which can synthesize methionine and cystine from sodium sulfate. The present studies were undertaken to augment the data on cystine metabolism in insects and to obtain information on the metabolism of sulfate, taurine, and methionine in the house fly.

MATERIALS AND METHODS

Administration of labeled compounds. Adult house flies, *Musca domestica* L., CSMA[4] strain, were used throughout these experiments. These flies

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[2] Present address: Bristol-Myers Products Division, Research Laboratories, Hillside, New Jersey.

[3] Present address: Chemicals and Plastics Division, U. S. Army Quartermaster Research and Engineering Center, Natick, Massachusetts.

[4] A DDT-susceptible strain of flies distributed by the Chemical Specialty Manufacturers Association.

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were routinely maintained on one-fourth strength skim milk. In tracer feeding experiments the flies, confined in Fernbach flasks (13), ingested an amino acid solution comparable to approximately 0.75 per cent casein hydrolyzate (6, p. 490) but lacking methionine and cystine. The solution also contained 5 per cent glucose and 0.1 per cent NaCl. S^{35}-labeled cystine, methionine, taurine or sulfate was added to the basic nutrients and the flies were permitted to feed for 22 to 24 hours.

Flies were injected (8) with 1 μl. of a solution containing labeled methionine or cysteine. Groups of flies were sacrified at various timed intervals after injection and prepared for chromatography. Each experiment was repeated several times. Extracts of the cysteine-injected flies were prepared at 1, 3, 5, 18, 21, and 24 hours after administration of the labeled compound.

Extraction procedure. The live flies were homogenized in 8 ml. of absolute alcohol containing an excess of *N*-ethylmaleimide. The volume was adjusted with water to 10 ml. of 80 per cent ethanol and the soluble fraction collected as described previously (15). This fraction was dried *in vacuo* and redissolved in 1 ml. of 10 per cent 2-propanol.

The extracted residues in the $S^{35}O_4$ and taurine-S^{35} feeding experiments were examined chromatographically for possible incorporation of S^{35} into the insoluble fraction. These residues were dried in a vacuum desiccator for several days after which they were sealed in ampules with 15 ml. of 6*N* HCl and hydrolyzed by autoclaving for 16 hours at 15 pounds of pressure. The hydrolyzates were filtered and evaporated to dryness on a steam bath. The solid material was dissolved in water and reduced to dryness several times. It was then dissolved in 1 ml. of 10 per cent 2-propanol.

The feces and regurgitum from flies fed $S^{35}O_4$ and taurine-S^{35} were washed out of the confining flasks, filtered, and the soluble material evaporated to dryness. This was then dissolved in 1 ml. of 10 per cent 2-propanol.

Chromatography. To separate the sulfur-containing compounds in the various preparations it was necessary to use a high-resolution paper chromatographic system. The solvents employed for development of the chromatograms were methanol-pyridine-water (20:1:5) (v/v) (16) and tertiary butyl alcohol-formic acid (88 per cent)-water (70:15:15) (v/v) (6). The first solvent took approximately 9 hours to migrate 50 cm. and the second approximately 20 hours to run 39 cm. at 25° C. by the descending method. The positions for a number of sulfur compounds based on Rf values are shown in Figure 1. Thiols were reacted with *N*-ethylmaleimide because the free thiols streaked badly (11). The positions of the *N*-ethylmaleimide adducts were sometimes checked using the method described by Benesch *et al.* (3).

Most of the sulfur-containing compounds were detected with ninhydrin.

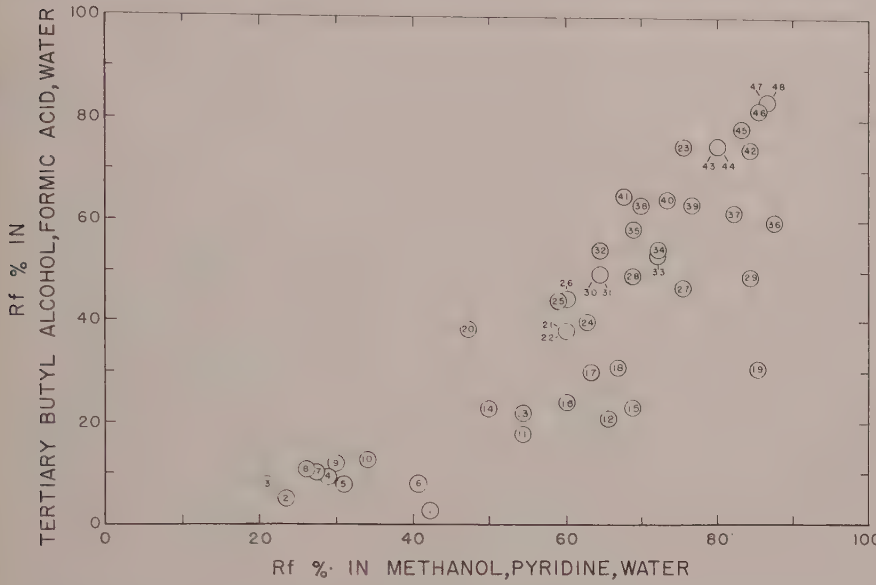


FIGURE 1. A two-dimensional chromatographic map of some sulfur compounds of biological importance. (26) *N*-Acetylglutathione, (20) *N*-acetylglutathione—*N*-ethylmaleimide adduct, (46) *N*-acetylhomocysteine thiolactone, (1) coenzyme A, (15) cystamine, (8) cystathionine, (33) cysteamine, (37) cysteamine—*N*-ethylmaleimide adduct, (10) cysteic acid, (29) cysteine ethyl ester hydrochloride, (42) cysteine ethyl ester—*N*-ethylmaleimide adduct, (34) cysteine—*N*-ethylmaleimide adduct, (14) cysteinesulfinic acid, (4) cystine, (5) *meso*-cystine, (7) djenkolic acid, (17) ergothioneine, (27) ergothioneine—*N*-ethylmaleimide, (43) ethionine, (30) ethionine sulfone, (38) *S*-ethylcysteine, (28) glutamylcysteine—*N*-ethylmaleimide adduct, (2) glutathione (oxidized), (25) glutathione—*N*-ethylmaleimide adduct, (16) homocysteine, (40) homocysteine—*N*-ethylmaleimide adduct, (23) homocysteine thiolactone, (45) α-hydroxy-γ-(methylmercapto)butyric acid, (18) hypotaurine, (19) isethionic acid, (3) lanthionine, (47) 2-mercaptoethanol, (48) β-mercaptopropionic acid, (39) methionine, (22) methionine sulfone, (21) methionine sulfoxide, (13) methionine sulfoximine, (32) *S*-methylcysteine, (11) methyl methionine sulfonium chloride, (6) sodium sulfate, (36) sodium taurocholate, (12) taurine, (41) β-2-thienylalanine, (9) thiolhistidine, (24) thiolhistidine—*N*-ethylmaleimide adduct, (44) thiomalic acid, (35) thiouracil, (31) thiourea.

Compounds possessing an amino but no carboxyl group, e.g., taurine, cystamine, etc., react with ninhydrin under alkaline or nearly neutral conditions so that chromatograms often had to be treated with ammonia vapors. A modification of the iodoplatinate reagent of Winegard *et al.* (19), (prepared by mixing 32 ml. of $0.002M$ H_2PtCl_6, 2 ml. of $1N$ KI, 3.2 ml. of $2N$ HCl, and 304 ml. of acetone) was used to detect some of the compounds which did not react with ninhydrin. With the iodoplatinate reagent most of the sulfur compounds appeared as white spots against a pink background; however, several compounds gave unusual color effects. Thiolhistidine and β-mercaptopropionic acid gave yellow spots. Ergothioneine gave a yellow spot with a dark grey border and its *N*-ethylmaleimide adduct gave a solid grey spot. Thiouracil gave the usual bleaching reaction at first and a bright cherry-red spot appeared in the bleached area 24 to 48 hours later. Chromatograms of extracts of several kinds of biological material indicated that there are several other compounds which may react with the platinic iodide reagent to produce yellow to grey spots. The presence of pyridine adversely affected the reaction. Both ninhydrin and iodoplatinate were applied by dipping the papers in a trough containing the reagent.

Taurocholate was detected by cutting the chromatograms into narrow bands and applying a modification of the Gregory and Pascoe test (12, p. 377): to a small test tube, 1 ml. of 45 per cent sulfuric acid and 1.7 ml. of 0.3 per cent furfural solution were added. A 1-cm. transverse band or its eluate from a single-dimensional run was then added, the tube stoppered loosely and heated to 65° C. for 20 to 30 minutes. A blue color indicated the presence of this bile acid. Fifteen to 30 μg. of sodium taurocholate could be detected. Chondroitin sulfate was detected with 0.04 per cent toluidine blue in 80 per cent acetone (10). This compound is not indicated in Figure 1 since it remains at the origin in both solvents.

Some compounds were degraded during chromatography. Cystine disulfoxide was almost completely converted to cystine and cysteinesulfinic acid. Cystamine disulfoxide was converted into hypotaurine during chromatography in the two-dimensional system but was largely intact after one-dimensional chromatography in the tertiary butyl alcohol-formic acid-water solvent. Hypotaurine (2-aminoethanesulfinic acid) was converted to taurine to a small extent. Methionine was partly converted to methionine sulfoxide in both solvents. Therefore, the two-dimensional chromatograms contained two methionine sulfoxide spots. The partial conversion of cystine to cysteinesulfinic acid was observed only when excessive quantities were chromatographed.

Dr. Claude Fromageot of l'Université de Paris kindly contributed cystine disulfoxide and hypotaurine. Dr. D. Cavallini of the Universita di Roma provided cystamine disulfoxide. Glutamylcysteine was prepared as previously described (9) and isethionic acid-S^{35} was prepared by the deamination of taurine-S^{35} (2, p. 13).

Radiometry. One-dimensional chromatograms, developed with the tertiary butyl alcohol-formic acid-water solvent were scanned for radioactivity with a strip scanner built for this laboratory by the Nuclear-Chicago Company, Chicago, Ill. Chromatograms were attached to a paper tape and drawn past a Micromil window on a D-47 gas flow detector by means of a flexible shaft attached to a recorder. The output of the analytical rate meter was fed into an Esterline-Angus recorder. The records obtained with this appratus were used to determine the S^{35} distribution on the chromatograms. The relative radioactivity of each peak was then determined by measuring the underlying area. Identities of the compounds responsible for the various peaks were ascertained by two-dimensional chromatography of the extracts and co-chromatography. Autoradiograms were prepared by placing the two-dimensional chromatograms in contact with Kodak No-Screen X-ray film for one month.

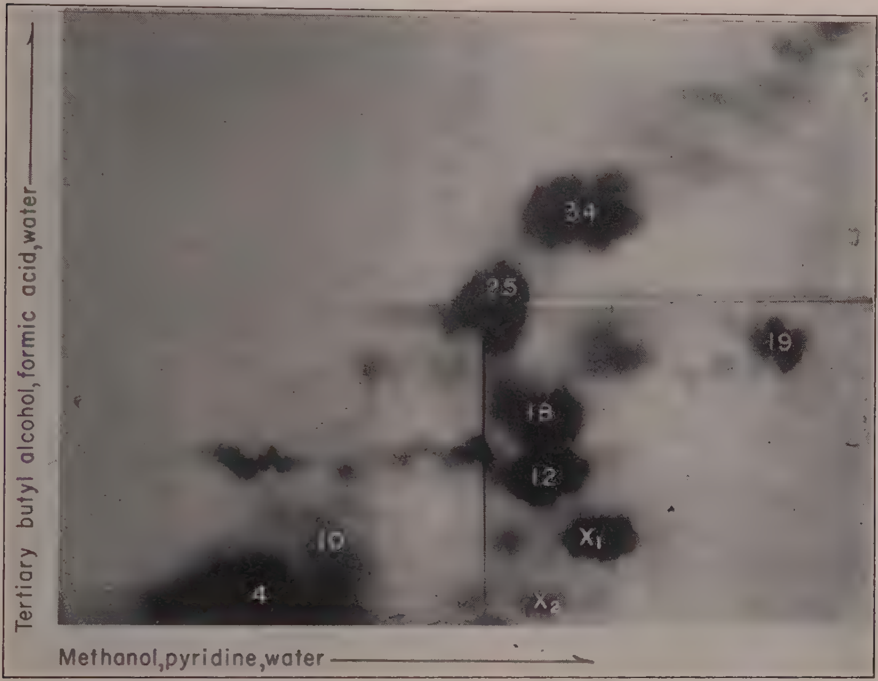


FIGURE 2. Autoradiogram of a two-dimensional chromatogram prepared from an extract of cystine-S^{35} fed house flies. (34) Cysteine—*N*-ethylmaleimide adduct, (25) glutathione—*N*-ethylmaleimide adduct, (18) hypotaurine, (12) taurine, (4) cystine, (10) cysteic acid, (19) possibly isethionic acid, (X_1, X_2) unidentified compounds.

RESULTS

Cystine and its oxidation products. In a typical cystine feeding experiment, 50 female flies were given 50 μc. of DL-cystine-S^{35} (specific activity 73.64 μc./mg.) in 2 ml. of nutrient solution. Twenty-four hours later an extract was prepared from the flies and chromatographed. Figure 2 illustrates an autoradiogram prepared from the chromatogram. The S^{35} was incorporated into a number of compounds including hypotaurine. The latter, present in a small quantity but of high specific activity, did not appear as a ninhydrin spot unless carrier was added. Figure 3 shows the distribution of S^{35} along a one-dimensional chromatogram. The absence of

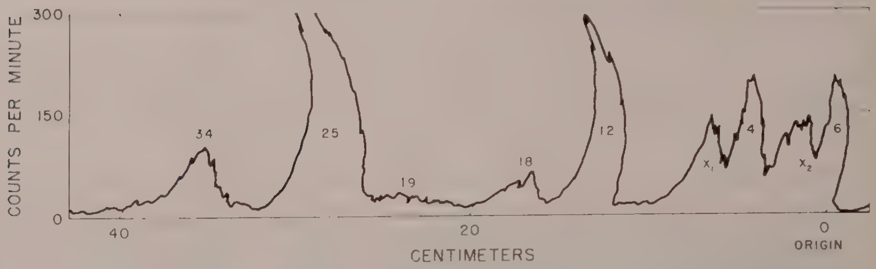


FIGURE 3. Distribution of S^{35} along a one-dimensional chromatogram prepared from an extract of cystine-S^{35} fed house flies; developed in tertiary butyl alcohol-formic acid-water. (34) Cysteine—*N*-ethylmaleimide adduct, (25) glutathione—*N*-ethylmaleimide adduct, (18) hypotaurine, (12) taurine, (4) cystine, (6) sulfate, (19) isethionic acid, (X_1, X_2) unidentified compounds.

radioactivity in the cystamine disulfoxide area on the chromatogram indicates that hypotaurine does not result from the chromatographic degradation of cystamine disulfoxide. Labeled cysteine, glutathione, cysteic acid and several unidentified sulfur compounds also appear in flies fed cystine-S^{35}. The possibility that microorganisms were responsible for some of the radioactive compounds observed was investigated by repeating the cystine feeding under aseptic conditions (8). Figure 4 shows a record obtained from a one-dimensional chromatogram of an extract of aseptic house flies fed cystine-S^{35} for 24 hours before sacrifice. It was recorded at high sensitivity to make minor components obvious. The record obtained from aseptic flies contained the same radioactive compounds as the record from flies reared without special aseptic precautions. Little or no cysteic acid was detected in the feeding experiments described above.

Extracts of flies injected with cysteine-S^{35} contained labeled metabolites

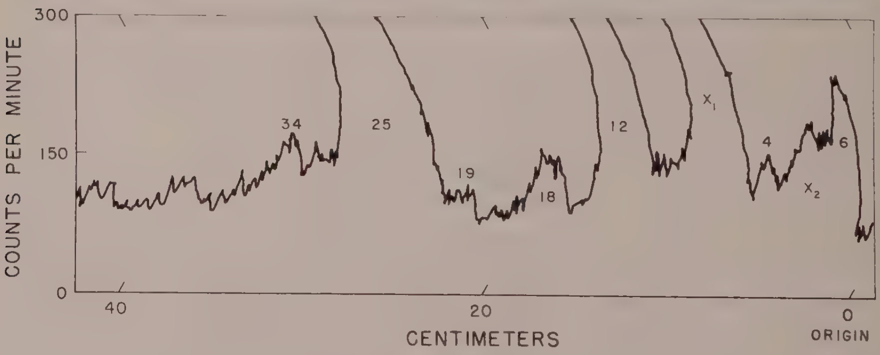


FIGURE 4. Distribution of S^{35} along a one-dimensional chromatogram prepared from an extract of cysteine-S^{35} fed aseptic house flies; developed in tertiary butyl alcohol-formic acid-water. (34) Cysteine—*N*-ethylmaleimide adduct, (25) glutathione—*N*-ethylmaleimide adduct, (18) hypotaurine, (12) taurine, (4) cystine, (6) sulfate, (19) isethionic acid, (X_1, X_2) unidentified compounds.

not observed in the feeding experiments. Figure 5 reproduces an autoradiogram of a chromatogram of such an extract prepared two hours after injection. There are spots corresponding to sulfate, cysteinesulfinic acid and an unknown which corresponds in Rf value to the *N*-ethylmaleimide adduct of cysteamine. Sulfate-S^{35} was found in extracts one and two hours after injection. Cysteinesulfinic acid and cysteamine were seen only in the samples taken two hours after injection.

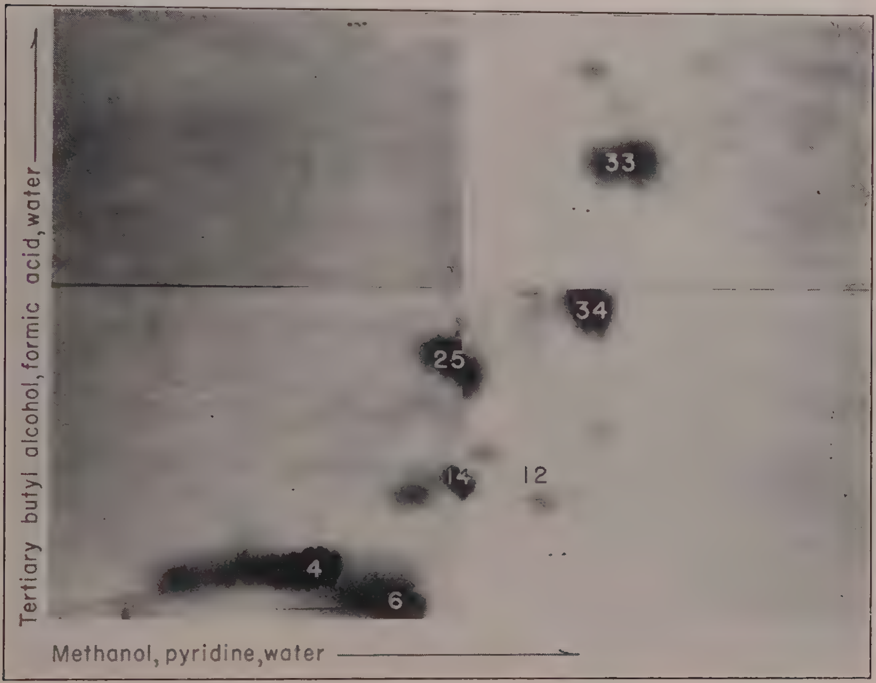


Figure 5. Autoradiogram of a chromatogram prepared from an extract of cysteine-S^{35} injected house flies. (34) Cysteine—*N*-ethylmaleimide adduct, (4) cystine, (25) glutathione —*N*-ethylmaleimide adduct, (6) sulfate, (12) taurine, (14) cysteinesulfinic acid, (33) cysteamine—*N*-ethylmaleimide adduct.

Sulfate-S^{35} was the only radioactive substance detected in the combined feces and regurgitum and in extracts of house flies which had been fed $S^{35}O_4$ in nutrient solution. The autoradiogram prepared from the chromatographed hydrolyzate of these flies showed a faint spot in the position of sulfate.

Chromatographic analysis of an extract of 40 female house flies fed 55 μc. of taurine-S^{35} (22 μc./mg.) indicated the presence of labeled taurine and two other compounds. One of these falls in the area corresponding to isethionic acid; the other is identical in chromatographic position with X_1 in Figure 2, which also shows a radioactive spot in the isethionic acid area.

Attempts to isolate X_1 from chromatograms were unsuccessful since the compound largely decomposed during the isolation procedure yielding taurine. Isethionic acid is not found in the feces of taurine fed flies, but X_1 and another unidentified compound, X_2, seen also in Figure 1 are present. Taurine was the only radioactive compound found in the hydrolyzed protein fraction of taurine-S^{35} fed flies.

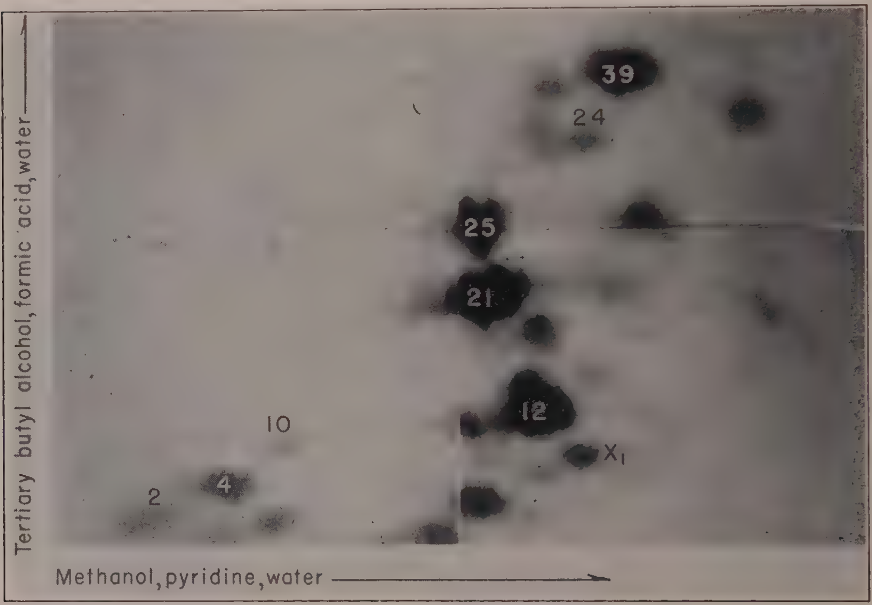


FIGURE 6. Autoradiogram of a chromatogram of an extract of methionine-S^{35} fed house flies. (39) Methionine, (34) cysteine—*N*-ethylmaleimide adduct, (25) glutathione—*N*-ethylmaleimide adduct, (21) methionine sulfoxide, (12) taurine, (10) cysteic acid, (4) cystine, (2) oxidized glutathione, (X_1) unidentified compound.

Methionine. The autoradiogram, prepared from a chromatogram of an extract of methionine-S^{35} fed house flies, is reproduced in Figure 6. Spots corresponding to methionine, methionine sulfoxide, cysteine—*N*-ethylmaleimide adduct, glutathione —*N*-ethylmaleimide adduct, oxidized glutathione, cystine, cysteic acid, taurine and a number of unidentified substances are present. A somewhat similar autoradiogram prepared from an extract of house flies injected with 6 μc. of methionine-S^{35} (48 μc./mg.) is shown in Figure 7. In addition to the compounds indicated above there are a number of unidentified spots near the methionine area.

To ascertain the participation of cystathionine in methionine metabolism another injection experiment was conducted as above, but 20 minutes before sacrifice the flies were injected with 25 μg. of DL-cystathionine. The function of the injected cystathionine was to serve as a carrier to prevent appreciable loss of any S^{35}-labeled cystathionine produced from the methionine-S^{35} previously administered. Spots corresponding to cystine, cystathionine, and cysteic acid were observed on an autoradiogram from this preparation. Support for the identity of cystathionine was obtained by chromatographing a small amount of extract in which a few μg. of cystathionine were dissolved. The compounds in the cystine, cystathionine, cysteic acid area were resolved by permitting the chromatographic solvents to flow off the ends of the paper. A radioactive spot (Fig. 8) coincident with the ninhydrin spot for the co-chromatographed cystathionine was obtained. An autoradiogram of a chromatogram of feces of house flies injected with S^{35}-labeled methionine is illustrated in Figure 9. There are spots corresponding to sulfate, taurine, and possibly deamination products of methionine.

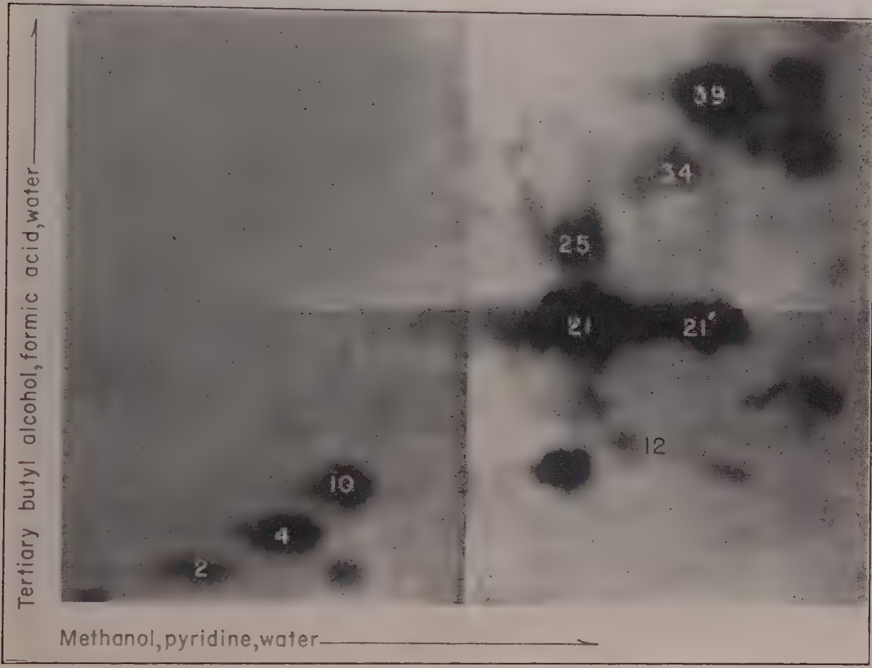


FIGURE 7. Autoradiogram of a chromatogram of an extract of house flies injected two hours previously with 6.26 μg. of methionine-S^{35} per fly. Specific activity 80 μc./mg. (39) Methionine, (21, 21′) methionine sulfoxide, (34) cysteine—*N*-ethylmaleimide adduct, (25) glutathione—*N*-ethylmaleimide adduct, (12) taurine, (10) cysteic acid, (4) cystine, (2) oxidized glutathione.

Feeding experiments provided data on the incorporation of S^{35} from methionine into glutathione and taurine. Simultaneous studies were made

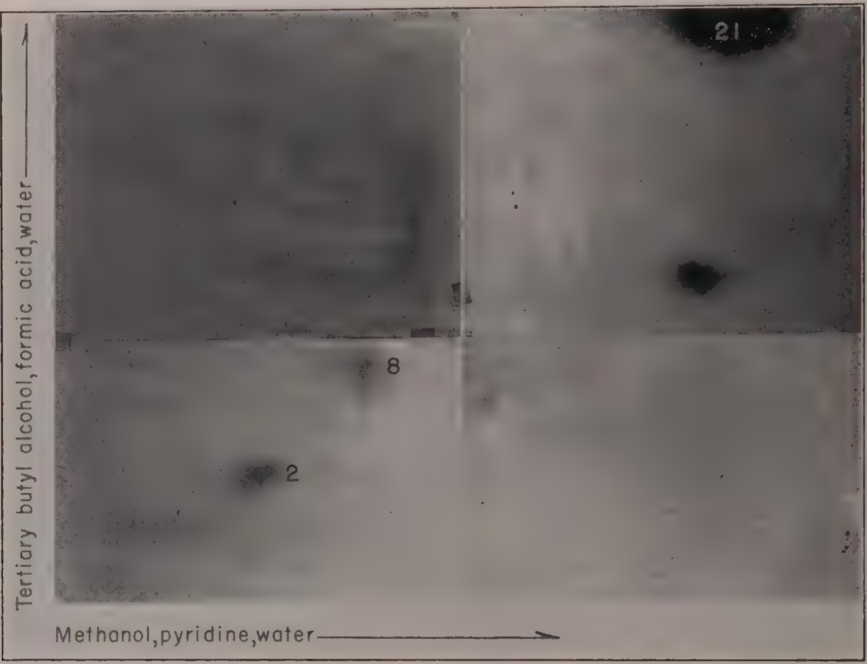

Tertiary butyl alcohol, formic acid, water
Methanol, pyridine, water
21
8
2

FIGURE 8. Autoradiogram of a chromatogram of an extract of house flies injected with 6 μg. of methionine-S^{35} per fly. Specific activity 48 μc./mg. One hour later the flies were injected with 25 μg. of DL-cystathionine and 20 minutes later were sacrificed and extracted. The chromatographic solvents in this case were permitted to flow off the ends of the paper to obtain greater resolution of spots. (8) Cystathionine, (2) oxidized glutathione.

with 1-day-old and 5-day-old female flies fed 50 μc. of DL-methionine-S^{35} (152 μc./mg.). A second series was set up to obtain comparative data using 50 μc. of DL-cystine-S^{35} (74 μc./mg.). After 22 hours each of the four groups of flies had consumed the same amount of fluid (1.6 ml.). The results are summarized in Table I.

TABLE I

INCORPORATION OF S^{35} INTO METABOLITES IN THE HOUSE FLY AFTER FEEDING CYSTINE-S^{35} OR METHIONINE-S^{35}

Age of flies in days	Labeled compound	Counts per minute per milligram live weight				Ratio Taurine / Glutathione
		Glutathione	Taurine	Fecal taurine	Fecal sulfate	
1	Cystine	441	231	42	612	0.52
5	Cystine	357	117	27	530	0.33
1	Methionine	54	115	Trace	24	2.13
5	Methionine	101	69	Trace	41	0.68

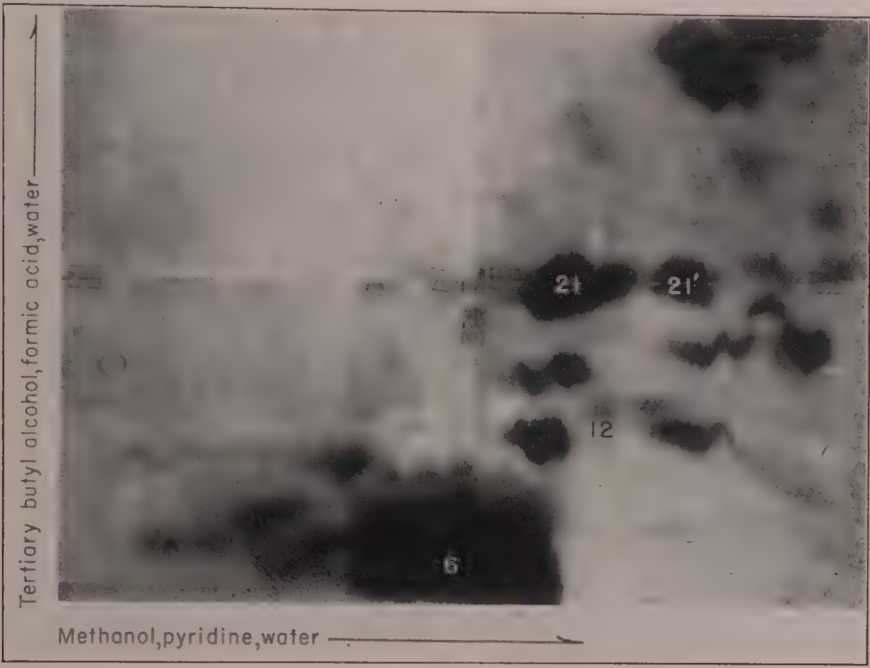


FIGURE 9. Autoradiogram of a chromatogram of feces from flies previously injected with 6 μg. DL-methionine-S^{35} per fly. Specific activity 80 μc./mg. (21, 21′) Methionine sulfoxide, (12) taurine, (6) sulfate. Many unidentified spots also appear.

DISCUSSION

The scheme below summarizes the results obtained after administration of various S^{35}-labeled compounds to house flies.

Cystine $\xrightarrow{\text{fed}}$ cysteine, glutathione, cystine, cystine disulfoxide, hypotaurine, taurine, sulfate, possibly isethionic acid, unidentified compounds X_1, X_2.

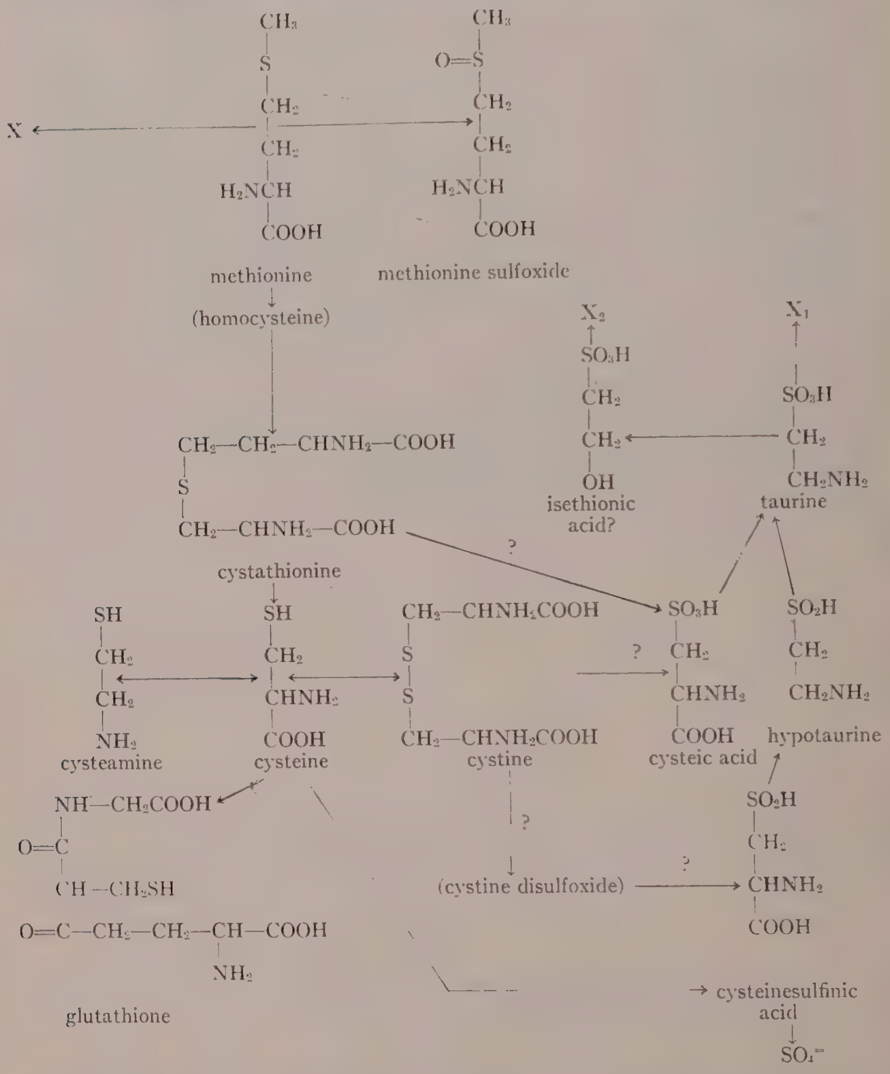
Cysteine $\xrightarrow{\text{injected}}$ cysteine, glutathione, cystine, cysteinesulfinic acid, possibly cysteamine, taurine, sulfate.

Taurine $\xrightarrow{\text{fed}}$ taurine, possibly isethionic acid, X_1, X_2.

Methionine $\xrightarrow[\text{injected}]{\text{fed}}$ methionine, methionine sulfoxide, cysteine, glutathione, cystine, cystathionine, cysteic acid, taurine, several unidentified compounds.

Sulfate $\xrightarrow{\text{fed}}$ sulfate.

The proposed interrelationships of these compounds as deduced from the above information may be schematically diagrammed as follows:



The compounds in parentheses above were not actually detected in the house fly. The probability of their occurrence, however, merits their inclusion in this scheme.

The primary pathway for the synthesis of taurine from cystine in the house fly appears to be via hypotaurine as an intermediate. Moreover, when methionine replaces cystine as the precursor, a great increase in cysteic acid is observed. The absence of cysteinesulfinic acid in this case indicates that cysteic acid is formed directly from either cystathionine or cystine. The appearance of cysteic acid as a precursor in taurine synthesis is not unlikely and has indeed already been demonstrated in other organisms (17). In the experiments in which both methionine-S^{35} and cystathionine were injected, the quantities of labeled cystine and cysteic acid produced were small compared to those formed after the injection of methionine-S^{35} alone. It is probable that this is primarily a dilution effect produced by increased concentrations of nonradioactive metabolites of cystathionine. However, it is also possible that the exogenous cystathionine resulted in retardation of methionine metabolism.

The conversion of taurine to several other compounds appears to be novel and offers partial explanation for the retention of relatively large amounts of taurine in the house fly (15). Further explanation awaits the identification of the taurine metabolites.

Cysteinesulfinic acid is a key intermediate in sulfate formation (1, 7). This compound is readily converted in mammals to hypotaurine and sulfate (4, 5, 17). It is probable that the cysteinesulfinic acid observed in these experiments was present as such in the fly extracts rather than being simply the result of chromatographic degradation of cystine disulfoxide.

The presence of $S^{35}O_4^=$ and taurine-S^{35} in the insoluble material after extraction of flies fed these substances might, of course, be due to incomplete extraction with 80 per cent ethanol. However, it is possible that they were incorporated into a constituent of the insoluble fraction. For example, $SO_4^=$ may become associated with a polysaccharide as a sulfuric acid ester. It is evident, however, that house flies are unable to synthesize significant amounts of sulfur amino acids from either taurine or sulfate since in none of the experiments in which these labeled compounds were fed was labeled cystine or methionine found. It may be concluded that house flies differ considerably in their sulfur metabolism from *Blattella* (14).

The data in Table I indicate that methionine-sulfur is rather quickly converted into glutathione-sulfur and taurine-sulfur in the house fly. This is in agreement with the results of Tabachnick and Tarver (18) in the rat. It is obvious that cystine is metabolized at a higher rate in 1-day-old flies than in 5-day-old flies. The same conclusion can not be drawn from the methionine data. Although taurine synthesis predominates, less sulfate is found in the feces of 1-day-old flies. It appears that when methionine is the precursor, older flies either synthesize more glutathione or retain this thiol longer than do young flies. The last column in Table I indicates that relatively larger amounts of taurine were synthesized when methionine was the precursor.

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GLUTATHIONE METABOLISM IN DDT RESISTANT AND SUSCEPTIBLE HOUSE FLIES[1]

VAL F. COTTY[2] AND S. MARK HENRY

SUMMARY

Glutathione concentration was measured in prepupae, pupae and newly emerged susceptible and DDT resistant house flies through its adduct with C^{14}-labeled *N*-ethylmaleimide. Free glutathione in both strains followed the same general pattern, no over-all difference being noted.

During the puparial period in the house fly the glutathione concentration decreased during the first two days, showed a relative increase on the third, and fell to a low level on the fourth day. The newly emerged adult fly had a higher level of glutathione than any other stage studied.

Measured amounts of S^{35}-labeled cysteine were injected into DDT resistant and susceptible house flies. At timed intervals the insects were killed and the glutathione was coupled with *N*-ethylmaleimide and isolated chromatographically for radioassay of S^{35}. Susceptible flies appeared to incorporate injected cysteine into glutathione and to metabolize the newly synthesized glutathione more rapidly than did resistant flies.

INTRODUCTION

The speculations of Fink (7), that insects tolerant towards arsenicals utilize glutathione (GSH) as a receptor for this heavy metal, have received support from the observations of Forgash. The latter has shown that injected GSH protects the American cockroach, *Periplaneta americana* L., against the toxic effects of arsenic trioxide and some other insecticides and that endogenous GSH decreases in this insect after arsenic poisoning (8). In a more recent publication (9) Forgash stated that the available supply of GSH in an insect is an index of the potential tolerance of that insect to arsenic. A possible relationship of GSH to DDT [2, 2-bis (*p*-chlorophenyl)-1, 1, 1-trichloroethane] tolerance was shown by Sanborn (16) who found a higher free sulfhydryl concentration in five DDT resistant strains of house flies than in a susceptible strain. A more direct relationship appeared with the report that the enzyme responsible for dehydrochlorination of DDT in house flies requires GSH for *in vitro* activation (17, 18).

The present studies were undertaken to determine whether GSH is primarily responsible for the elevated sulfhydryl level in resistant flies and to study the synthesis and retention of GSH by the house fly.

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[2] Present address: Bristol-Myers Products Division, Research Laboratories, Hillside, New Jersey.

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MATERIALS AND METHODS

Gluthathione Concentration during Different Stages of Development

The two strains of house flies, *Musca domestica* L., employed in this study were the CSMA[3] and the DDT-45[4] strains. Adult stocks of the resistant strain were kept in a cage coated with DDT to maintain a high degree of resistance. Full grown larvae were removed from the rearing medium (13) as soon as they became white prepupae. The prepupae were either used immediately or the date and time of collection were recorded and the insects were incubated at 32° ± 1° C. at a high humidity for later use. The age of the pupae at harvesting was timed with an accuracy of ± 15 minutes. Pupae were collected from two generations. All values are averages of triplicate determinations.[5]

Hanes *et al.* (12) showed that *N*-ethylmaleimide could be used to convert sulfhydryl compounds into stable derivatives for chromatography. Gutcho and Laufer (11) studied the behavior of glutathione and related compounds in a number of solvent systems and suggested the use of C^{14}-labeled *N*-ethylmaleimide for GSH determinations.

In making the determinations the following procedure was used: timed groups of pupae, averaging 11 individuals, were weighed and homogenized with a Ten Broeck tissue grinder in 3 ml. of 33 per cent (v/v) ethanol containing 2 mg. of C^{14}-labeled *N*-ethylmaleimide. The 33 per cent ethanol replaced absolute ethanol used in previous work in order to assure complete extraction of alcohol-insoluble compounds. The homogenate was adjusted to 80 per cent (v/v) ethanol by adding absolute ethanol, and the insoluble material was separated by centrifuging. The supernatant solution was removed, and the residue extracted twice more with 10 ml. of 80 per cent ethanol. The three supernatant solutions were pooled and evaporated to dryness *in vacuo*. The solid matter was then dissolved in 0.5 ml. of water and extracted with 0.5 ml. of chloroform. One hundred μl. of the water-soluble concentrated extract were applied as a small spot to a sheet of Whatman No. 3 paper. A one-dimensional chromatogram was then developed in *tertiary*-butyl alcohol-formic acid (88 per cent)-water (70:15:15) (2, p. 91). The chromatogram was treated with ninhydrin and scanned for radioactivity with a recording gas flow scanner described in the accompanying paper (4). The record obtained from the scanner was used to determine the glutathione content. The area under the peak corresponding to glutathione—N-ethylmaleimide adduct was measured with a planimeter and converted to mg. of glutathione by dividing the area by the slope of a curve prepared with known amounts of C^{14}-labeled glutathione—N-ethylmaleimide adduct. Additional evidence for the identity of GSH was obtained by chromatographing the extracts in two other solvents. It should be noted that when N-ethylmaleimide reacts with GSH in 33 per cent ethanol a small amount of a second product, probably oxidized glutathione, is formed. The extent of the reaction was determined with the nitroprusside test (10) and chromatography. The amount of the second product was constant and the reaction went to completion very quickly. Since the procedure was standardized with pure glutathione, this second product need not be considered further. The C^{14}-labeled N-ethylmaleimide was supplied in an alcoholic solution. Stock solutions were prepared by adding carrier N-ethylmaleimide and diluting with ethanol to the required specific activity.

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[3] A DDT susceptible strain distributed by the Chemical Specialties Manufacturers Association.

[4] A DDT resistant strain obtained from Dr. Albert Perry of the United States Public Health Service, Savannah, Georgia.

[5] The authors are grateful to Dr. Frank Wilcoxon who provided statistical analyses of the data.

Gluthathione Synthesis and Degradation

Three DDT resistant strains and one susceptible strain were used in injection experiments to determine glutathione synthesis and degradation after cysteine-S^{35} administration. The CSMA (susceptible) and DDT-45 (resistant) strains were discussed previously. The additional resistant strains, designated ACC and DDT-2, were supplied as subcultures of the Ellenville strain by Dr. Charles Hasset of the Army Chemical Center, Maryland. The DDT-2 strain was found to have lost resistance to DDT. Resistance was restored by keeping the breeding stock of these flies in cages which were at first partially and later completely coated with DDT. Adult flies used in the actual determinations were not exposed to DDT at any time. They were fed one-fourth strength skim milk before and during the experiments. Parallel experiments were run with resistant and susceptible flies of the same age. Only adult female flies 3 to 6 days of age were used.

Since cystine is virtually insoluble in water at physiological pH levels, it was necessary to convert it to cysteine. DL-cystine-S^{35} (Volk Radiochemical Company, Chicago, Ill.) of high specific activity (up to 167 μc./ml.) in $2N$ HCl was reduced by adding an excess of L-cysteine hydrochloride under nitrogen and then bringing the solution to neutrality with $10N$ NaOH. In one case labeled cysteine was obtained by reducing L-cystine-S^{35} (Schwarz Laboratories, Mount Vernon, N. Y.). This was accomplished by first forming a mercaptide with mercuric acetate. The mercaptide was then decomposed with hydrogen sulfide to produce cysteine. This method was abandoned because of the loss of radioactivity from the use of hydrogen sulfide.

The flies were injected with a micrometer-controlled 1-ml. tuberculin syringe equipped with a microneedle. The microneedles differed from those described by Martignoni (14) in that the glass tips were drawn with a double taper to an outside diameter of 90 μ and were not fire polished. The apparatus with a fly placed in position for injection is illustrated in Figure 1. The syringe was filled with paraffin oil, the air bubbles were displaced and the cysteine solution was drawn into the syringe beneath the oil. The use of oil in this manner greatly reduced troublesome air bubbles and leaks. Each fly received 1 μl. of solution containing approximately 50 μg. of labeled cysteine except in one experiment in which each fly received 3 μg. of cysteine.

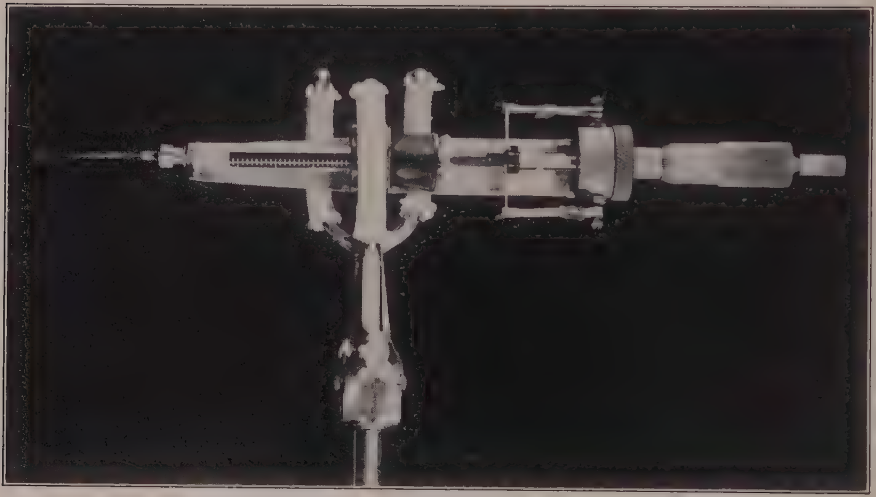

FIGURE 1. Apparatus for injecting house flies with accurately measured microquantities of fluid.

The intervals between injection and homogenization of the insects were timed with a variation of ±10 minutes. This time variation was due to the fact that the flies were injected in groups and the average time for a group was used. At the indicated intervals, groups of flies averaging 12 individuals were anesthetized with CO_2, weighed and homogenized in 3 ml. of 33 per cent ethanol containing 20 mg. of *N*-ethylmaleimide per 100 mg. live weight of house flies. Extracts were prepared as described above.

Glutathione-S^{35} –*N*-ethylmaleimide adduct was isolated chromatographically and measured radiometrically exactly as described above for the C^{14}-labeled compound. Taurine-S^{35} and cysteine-S^{35}—*N*-ethylmaleimide adduct were determined in a similar manner.

GSH was obtained from Nutritional Biochemicals Corporation and

L-cysteine hydrochloride and *N*-ethylmaleimide from Schwarz Laboratories, Inc. Dr. Max Laufer of Schwarz Laboratories very kindly contributed the C^{14}-labeled *N*-ethylmaleimide. The latter compound had a specific activity of 3.23 μc./mg. and contained an impurity accounting for 16 per cent of the radioactivity. The radioactive impurity moved at the front during chromatographic development. The C^{14}-labeled *N*-ethylmaleimide was diluted with carrier to a specific activity of 1 μc./mg. and stored in 95 per cent ethanol at −8° C.

RESULTS

Changes in the concentration of GSH during the puparial period are plotted in Figure 2. In this figure the values for free GSH are based on live weight of the insects. Curves determined by plotting concentration of GSH per individual insect or per mg. dry weight of extracted residue against time follow the same general pattern. Statistical analyses of the data for the three sets of curves indicated a significant over-all difference at the 5 per cent level between the two strains in only one case (based on live weight). However, all three sets of curves show a similar age response.

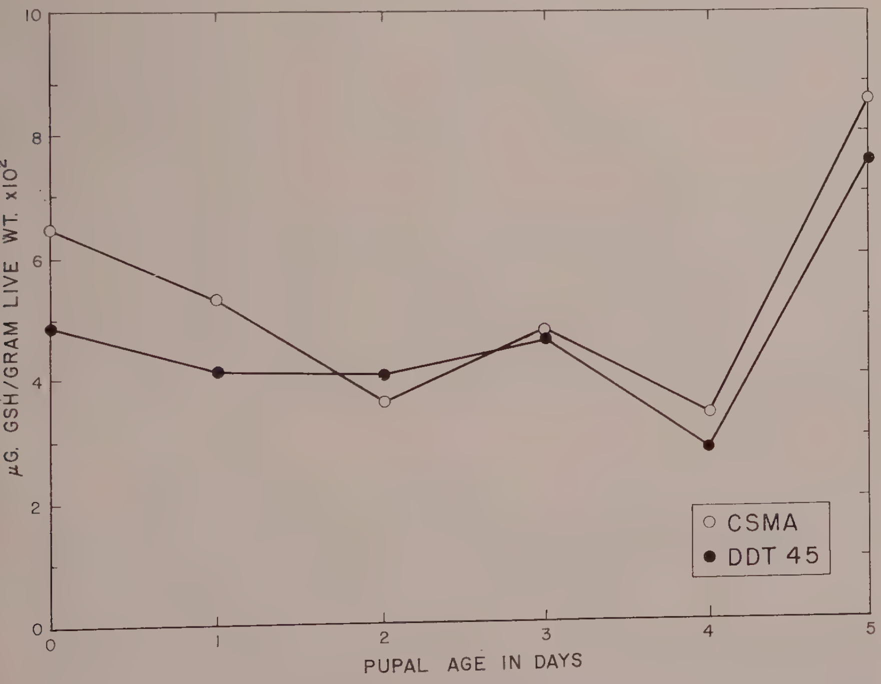

Figure 2. Changes in glutathione concentration in DDT resistant (DDT-45) and susceptible (CSMA) house flies during the puparial period beginning with white prepupae (0 days) and ending with newly emerged adults (5 days).

TABLE I

CHANGES IN CONCENTRATIONS OF GLUTATHIONE-S^{35} SYNTHESIZED IN RESISTANT AND SUSCEPTIBLE ADULT HOUSE FLIES INJECTED WITH L-CYSTEINE-S^{35}

Resistant strain used	Injection data	Time after injection (hours)	Counts/min./mg. live wt.		Ratio $\frac{(S)}{(R)}$
			Susceptible (S)*	Resistant (R)	
DDT-45	3 μg. L-cysteine-S^{35} per fly	3	192	219	0.9
		5	150	207	0.7
		24	46	93	0.5
DDT-45	0.54 μg. DL-cystine-S^{35} and 45 μg. L-cysteine per fly	1	237	117	2.0
		18	203	154	1.3
DDT-2	0.6 μg. DL-cystine-S^{35} and 37 μg. L-cysteine per fly	2	326	152	2.1
		21	232	144	1.6
ACC	0.6 μg. DL-cystine-S^{35} and 50 μg. L-cysteine per fly	3	106	118	0.9
		21	50	60	0.8
		25	35	60	0.6

* The CSMA (susceptible) strain was used in all cases.

Determination of the fourth degree coefficient indicated that the relative rise in GSH concentration on the third day was significant at the 1 per cent level. The curves may be generally described then as W-shaped. The newly emerged adult had a higher level of GSH than any other stage studied.

The records from an experiment in which cysteine-S^{35} (3 μg. per fly) was injected into adult house flies are shown in Figure 3. The data from these records appear in Table I. In each experiment the ratio of glutathione in susceptible flies to that in resistant flies decreased with time. Taurine-S^{35} ratios in these four experiments showed no consistent trend while cysteine-S^{35} ratios showed a trend opposite to that of glutathione. No qualitative differences in the sulfur metabolism of susceptible and resistant flies are apparent from these records.

DISCUSSION

The results of glutathione determinations in resistant and susceptible flies indicate that the higher sulfhydryl concentration of adult resistant flies as determined by Sanborn (16) may be due to compounds other than glutathione. Other sulfhydryl compounds were detected during the present studies but no attempt was made to measure these quantitatively. No outstanding differences in glutathione concentration between the two strains at different ages were noted. Unfortunately, no data on glutathione are available for what is perhaps the most significant part of the metamorphic period considered in relation to DDT resistance, viz., the period

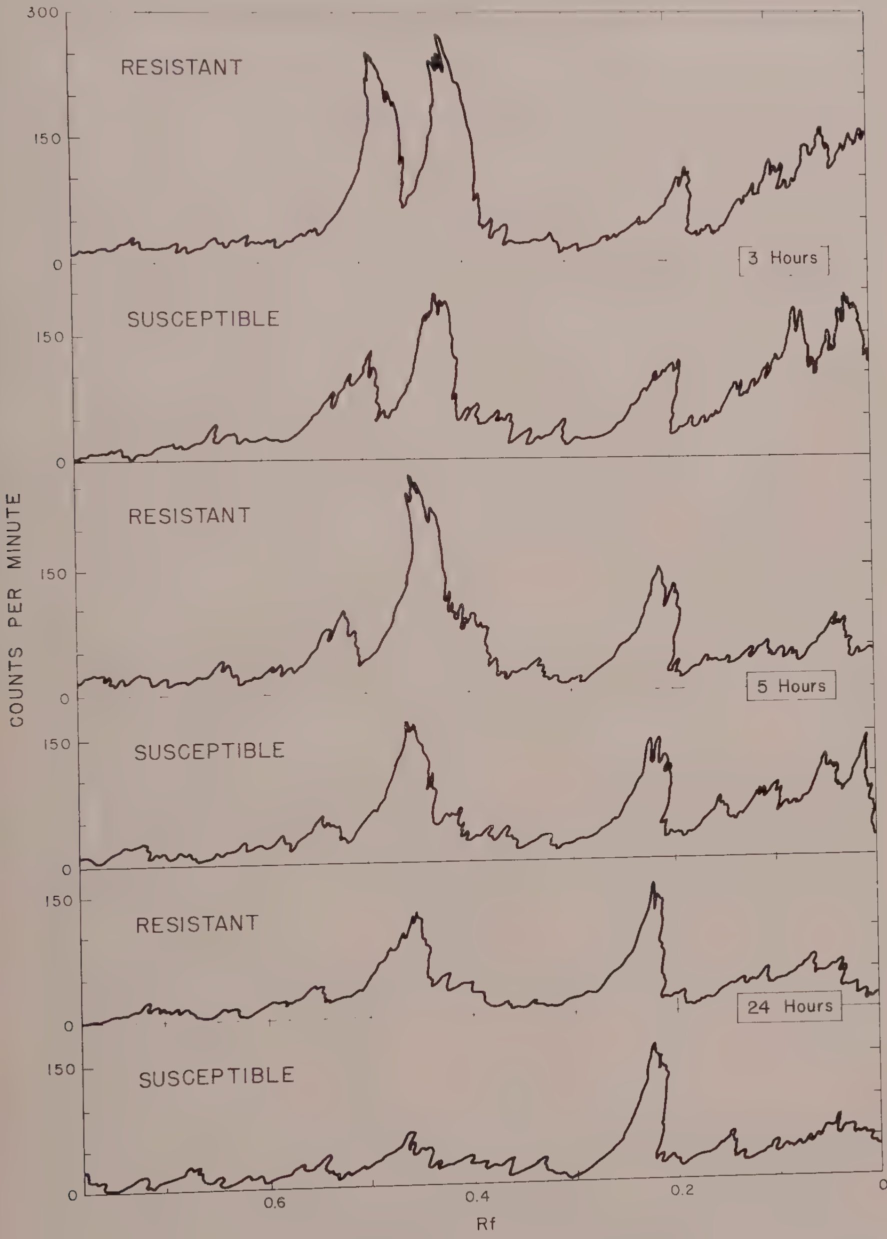

Figure 3. The distribution of S^{35} on unidimensional chromatograms of extracts from resistant (DDT-45) and susceptible (CSMA) house flies at different times after the injection of 3 μg. of cysteine-S^{35}. The most conspicuous components are cysteine—*N*-ethylmaleimide adduct (Rf 0.54), glutathione—*N*-ethylmaleimide adduct (Rf 0.44), and taurine (Rf 0.21).

just preceding the prepupal stage, when the DDT-detoxifying enzyme is at its highest level (15).

The curve described by the changes in GSH level is not exactly similar to that for any of the metabolic or physiological functions reported for the house fly (3, 5, 6). The synthesis of GSH requires adenosine triphosphate and is strongly inhibited by adenosine diphosphate (19). Agrell (1) reported variations in ATP and ADP levels during the puparial period in the blow fly. If such changes also occur in the house fly, these may constitute one of the factors determining GSH level.

The data in Table I are compiled from four separate experiments which were not replicated. The four experiments were nevertheless not greatly dissimilar. The amount of cysteine injected is radically different in only one experiment. The radioactivity of the preparations was, however, quite variable and the ages of the flies, although the same for any one experiment, differed slightly between experiments. These differences are probably responsible for the variation observed in the amount of $GS^{35}H$ synthesized by the susceptible strain in these experiments.

In the second experiment (Table I) the amount of $GS^{35}H$ present in the resistant flies appears to increase between 1 and 18 hours while in all other experiments there is a decrease with time. It should be noted that the readings in this experiment were taken only one hour after injection, when synthesis of GSH probably was still occurring at a fast rate and the maximum incorporation of cysteine-S^{35} therefore was not yet attained. The same explanation may be offered for the apparently greater glutathione degradation by ACC flies as compared to DDT-2 flies.

The data in Table I indicate that susceptible flies injected with cysteine-S^{35} synthesize $GS^{35}H$ more rapidly than do resistant flies. Moreover $GS^{35}H$ in resistant flies is degraded less rapidly than in susceptible flies. These conclusions are based on recurrence of the same trend (S R ratio, Table I) with different resistant strains and with variations in procedure. It is suggested that the lower rate of GSH degradation in resistant flies is partially a result of metabolic stabilization, perhaps by the DDT-detoxifying system.

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THE ORIGIN OF DDT RESISTANCE IN THE HOUSE FLY

Herbert H. Moorefield

SUMMARY

The origin of DDT resistance in the house fly, in terms of a metabolic phenomenon, cannot be described as a *de novo* synthesis. The detoxication enzyme, DDT-dehydrochlorinase, which has previously been shown to be correlated with the presence of resistance in the adult fly, has now been demonstrated in the larvae of susceptible strains that have no history of exposure to DDT.

Experiments, designed to learn if the concentration of enzyme could be increased within a single generation of flies, were performed to test the possibility that adaptation may be involved in this type of insecticide resistance. Sublethal levels of DDT applied topically to adult susceptible flies failed to produce a demonstrable level of DDT-dehydrochlorinase. Further, the intensity of detoxication in the larvae could not be augmented by incorporating DDT or nontoxic analogues of DDT which have an affinity for the enzyme into the medium in which the maggots were reared. The fact that an increased potential to metabolize DDT could not be produced without selection, indicates that the development of DDT-dehydrochlorinase is not an enzyme induction process, and the genetic implications in origination of resistance in the house fly are substantiated.

INTRODUCTION

The development of resistant organisms has serious implications and has imposed severe limitations on the use of modern pesticides, drugs and antibiotics. The question of how these tolerant strains and species arise has been the subject of investigation of workers in many different disciplines, and symposia consolidating researches in various specialized fields have aided in orienting the experimental data relating to this phenomenon (7, 19). Observations on the origin of resistance can be conveniently classified into two general categories.

1. *Mutative or genetic adaptation.* Mutants may either arise spontaneously before exposure to the toxicant, or individuals may be produced through the action of a mutagenic chemical. The new genotypes, more readily adjusted to the adverse environment, are then subsequently selected out by the lethal action of the toxic agent. In time, the hardier progeny become dominant and substitute for the old, susceptible population.

2. *Inductive or physiological adaptation.* Specific reactions may be produced within the exposed organisms as a result of the presence of the toxicant. The hereditary mechanism remains unaltered and tolerance is acquired through the quantitative increase of a metabolic or detoxication system such as an adaptive enzyme.

Evidence has been presented in entomological studies that can be interpreted to support either or both pathways of development of insect resistance to chemicals. Significance of the genetical role in insecticide tolerance has been fully treated recently in comprehensive reviews (5, 13).

Although the predominant information upholds the mutation hypothesis, observations have also been reported that more closely satisfy the requirements defined by an inductive origin. Garin (8) claims to have produced bedbugs (*Cimex lectularis* L.) that were resistant to 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT), and 1,2,3,4,5,6-hexachlorocyclohexane (BHC) within a single generation by exposure to sublethal doses. Similarly, studies with the body louse (*Pediculus humanus* L.) have been cited which state that resistance to DDT was achieved through conditions that caused no apparent mortality or injury (6). Vaguely described results refuting the involvement of selection have also been published concerning the development of tolerance of house flies (*Musca domestica* L.) to Dimetan (5,5-dimethyldihydroresorcinol dimethylcarbamate) (3, pp. 75–76).

The finding that the resistant house fly could degrade DDT to a nontoxic compound, 1,1-dichloro-2,2-bis (*p*-chlorophenyl)ethylene (DDE) (20), was a significant contribution to the understanding of insect resistance. Later experimentation disclosed that this degradation was enzymatically catalyzed (22) and, more recently a direct correlation has been established between the presence of DDT tolerance in this species and the occurrence of the detoxication enzyme, DDT-dehydrochlorinase (21).

These researches have presented a factor which will reflect the degree of resistance in a given strain of flies and which can be quantitated independent of the use of dosage-mortality techniques (14, 15). The following study was undertaken to learn whether the DDT-dehydrochlorinase can be inductively increased within a single generation or if any physiological adaptation of this mechanism can be detected in the origin of DDT resistance in the house fly.

MATERIALS AND METHODS

The house flies used in this study were reared by the standard Peet-Grady method (16). Incorporation of chemicals into the larval medium was accomplished by crystallizing them from acetone solutions onto the dry bran used in preparation of the mash. Fly eggs were seeded into the mixture, and 4.5 days later, just prior to the time of pupation, the fully grown maggots were collected, washed with water, and powdered in acetone.

Acetone powder preparations were made by first grinding either adult flies or maggots with a VirTis homogenizer in 10 (w/v) equivalents of acetone, precooled to −10° C. The resulting slurry was filtered on a chilled Buchner funnel and the residue washed with cold ethyl ether. The filter cake, wet with ether, was transferred to a vacuum desiccator and the last trace of solvent removed under reduced pressure. Such preparations were free of the added test chemicals.

Assays of DDT-dehydrochlorinase activity were made by previously described methods (21). Two g. of powder were suspended in 10 ml. of 0.137*M* phosphate buffer (pH 7.4), and after steeping the mixture for one hour at 5° C. the insoluble fraction was filtered out through cheesecloth. Enzyme reactions were carried out in single sidearm Warburg vessels. The main compartment contained 4 mg. of substrate, DDT, crystallized from acetone onto glass beads (15 to 60 microns diameter), and 2.5 ml. of the enzyme preparation. Nitrogen was flushed through the flasks for 15 minutes, and the assays were carried out under anaerobic conditions. Zero time was marked by the introduction of 3 mg. of glutathione, contained in 0.5 ml. of buffer, from the sidearm. The reactions were run at 37° C. for 90 minutes. Quantitative analyses of the DDT and DDE were made by treating the reaction mixtures with 3 ml. of concentrated sulfuric acid, extracting with 8 ml. of cyclohexane, and measuring the absorbance of aliquots of the solvent layer at 241 and 260 mμ with a Beckman DU spectrophotometer. Standard calculations for two-component systems yielded the percentage of substrate that had been enzymatically dehydrochlorinated (21). Each determination was performed in duplicate, and each experiment was repeated at least three times.

Dosage-mortality measurements were made by topical administration of microdrops onto the mesonota of three-day-old adult female flies. The chemicals were contained in 1-μl. doses of acetone delivered from a micrometer-driven syringe. Mortality counts were made 24 hours after treatment.

RESULTS

The present experiments verify earlier investigations which have shown that both adults and larvae of DDT resistant strains of house flies have the capacity to metabolize this insecticide and that no detoxication can be demonstrated in adult susceptible flies (21). However, active DDT-dehydrochlorinase preparations have been derived from the larvae of these susceptible strains. Table I shows comparative relative concentrations of DDT-dehydrochlorinase found in immature and imaginal stages of both susceptible and resistant strains of the house fly. Equivalent preparations of larval and adult flies were made by simultaneously assaying equal aliquots of breis containing 200 insects homogenized in 20 ml. of cold, distilled water.

In order to determine if a physiological role could be involved in the development of DDT resistance four approaches were made attempting to inductively increase the level of the detoxication mechanism in the sus-

TABLE I

COMPARATIVE LEVELS OF DDT-DEHYDROCHLORINASE IN DDT-SUSCEPTIBLE AND RESISTANT STRAINS OF HOUSE FLIES

Strain	History	LD50-DDT μg./adult	μg. DDT-dehydrochlorinated *in vitro* by equivalent preparations	
			Adults	Larvae
CSMA (Susceptible)*	No previous exposure to DDT	0.25	0	240
C-155 (Susceptible)**	No previous exposure to DDT	0.3	0	255
"Synergist" (resistant)***	Selected with DDT+ $(p\text{-ClC}_6\text{H}_4)_2\text{CHCH}_3$+ $(p\text{-ClC}_6\text{H}_4)_2\text{CHCl}$ (14)	>50 (too high to measure)	440	720

* Obtained from Chemical Specialties Manufacturers Association.
** Obtained from Dr. L. Chadwick, Army Chemical Center, Maryland.
*** Obtained from Dr. R. B. March, Citrus Expt. Sta., Riverside, California.

ceptible fly. Topical, sublethal doses of DDT and nontoxic analogues of DDT were applied to adult flies. No significant activity of DDT-dehydrochlorinase could be detected in acetone powders of these insects prepared and assayed 72 hours later. Similarly, no increased titer of the enzyme could be found as a result of rearing susceptible house fly larvae under continuous exposure of sublethal quantities of DDT or analogues. The results of these various trials are presented in Table II.

The LD50 of susceptible adult flies (CSMA strain) reared in normal media was 0.25 μg./fly. A comparative LD50 of insects raised in DDT treated media was 0.08 μg./fly. This apparent sensitization has been reported previously (13).

DISCUSSION

The discovery of DDT-dehydrochlorinase in the larvae of susceptible house flies that have had no prior exposure to DDT is the first evidence that a *de novo* enzyme synthesis has not taken place in the resistant strains. The presence of this mechanism has been intimated by the work of other investigators (17, 20) who have previously demonstrated by chemical methods that the susceptible maggot can produce DDE. Chromatographic procedures have also been used to indicate the capacity of susceptible fly tissue to metabolize DDT (18).

It has been shown that in the life history of the tolerant fly, the maximum concentration of DDT-dehydrochlorinase is achieved in last instar larvae. The enzyme level falls to approximately one-half this quantity at the time of pupation and the resultant titer is maintained at a near con-

TABLE II

ATTEMPTED INDUCTION OF DDT-DEHYDROCHLORINASE IN SUSCEPTIBLE HOUSE FLIES

Experiment	Treatment	Compound used	Strain of flies	µg. DDT-dehydrochlorinated *in vitro* by equivalent preparations	
				Untreated	Treated
Adults treated topically with sublethal dose of toxicant	0.1 µg./fly applied. Acetone powders prepared 72 hrs. later	$(p\text{-ClC}_6\text{H}_4)_2\text{CHCCl}_3$	CSMA	0	0
			C-155	0	5
Adults treated topically with analogues of toxicant	3 µg./fly applied. Acetone powders prepared 72 hrs. later	$(p\text{-ClC}_6\text{H}_4)_2\text{CHCH}_3$	CSMA	5	5
			C-155	0	0
		$(p\text{-ClC}_6\text{H}_4)_2\text{CHCH}_2\text{OH}$	CSMA	5	0
			C-155	0	5
Larvae reared in environment of sublethal level of toxicant	80 p.p.m. of compound incorporated into media	$(p\text{-ClC}_6\text{H}_4)_2\text{CHCCl}_3$	CSMA	225	230
			C-155	230	215
Larvae reared in environment of analogues of toxicant	160 p.p.m. of compound incorporated into media	$(p\text{-ClC}_6\text{H}_4)_2\text{CHCH}_3$	CSMA	235	235
			C-155	240	250
		$(p\text{-ClC}_6\text{H}_4)_2\text{CHCH}_2\text{OH}$	CSMA	255	240
			C-155	235	250

stant amount for the remainder of the flies' life (15). If the pattern of relative enzyme concentration described for the various life stages of the resistant fly were indeed paralleled in the susceptible strains, the peak of intensity occurring in the late larvae may well be expected to be demonstrable by available enzyme assay techniques. However, the level in the adult susceptible fly may well be present and yet fall below the sensitivity of the method used in these studies.

The fact that DDT-dehydrochlorinase is not the result of a mutagenic property of DDT was implied when selection of house flies with toxicants other than chlorinated hydrocarbons, such as certain phosphates (2) and urethanes (12), were shown to increase the tolerance to DDT. Kerr *et al.* (11) definitely precluded the possiblity of mutation being caused by activity of the insecticide by showing an increased resistance to DDT, accompanied by a concomitant appearance of DDT-dehydrochlorinase in tolerant adults by merely selecting the late-emerging flies. The existence of the enzyme in larvae of fly populations that are susceptible, that have no previous experience of DDT, and that have not been purposely selected by any means suggests that to develop a resistant strain a mechanism must be established providing an extension of the detoxication potential from the larva, through the pupa, into the adult together with a means of quantitatively increasing the concentration of the enzyme. The function served by the DDT-dehydrochlorinase in the normal house fly is unknown.

Many unsuccessful attempts have been made to increase insect resistance to insecticides by exposure to sublethal concentrations of toxicant (1, 4, 9, 10, 23). The results of the present investigation are in accord with these findings and also show that the presence of DDT not only fails to supplement the tolerance of the flies, but it likewise does not influence the titer of DDT-dehydrochlorinase present in the exposed generation. Further, an intimate contact with nontoxic analogues of DDT, which have been reported to exhibit a high degree of affinity for this detoxifying enzyme as competitive inhibitors (14), will not effect any adaptation in the susceptible house fly.

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ABSORPTIOMETRIC ANALYSIS OF *N*-(TRICHLOROMETHYLTHIO)-4-CYCLOHEXENE-1,2-DICARBOXIMIDE (CAPTAN)

H. P. Burchfield and Joan Schechtman

SUMMARY

Captan can be analyzed by measuring the intensity of the color formed when it reacts with pyridine and tetraethylammonium hydroxide. The absorption maximum is 415 mμ, and the molar absorbance is 1.3×10^4 when calculated on the basis of the initial concentration of captan. Color stability is excellent in the presence of the quaternary base but very poor when sodium hydroxide is substituted for it. The related fungicide Phaltan [*N*-(trichloromethylthio)phthalimide] can be determined under identical conditions. Recoveries of 75 to 94 per cent were obtained when known amounts of captan were added to various media, indicating that part of the fungicide decomposes very rapidly. The color intensity produced by captan diminishes by one-half within 2.5 hours when solutions of it in pH 7.0 phosphate buffer are incubated at 29° C. for various times before adding the reagents. This shows that it hydrolyzes much more rapidly than some of the fungicides containing reactive halogen reported on previously.

INTRODUCTION

Captan can be determined colorimetrically by reactions with resorcinol (7, 11), but during studies on the persistence of a number of fungicides and related compounds in soils it was desirable to use comparable analytical methods for residue studies. Previous work done in these laboratories showed it was possible to analyze for a number of *s*-triazine derivatives (3), 1-fluoro-2,4-dinitrobenzene (6), and 3,4-dichlorotetrahydrothiophene-1,1-dioxide (1) by causing them to react with pyridine and alkali. Accordingly, an analytical method for captan based on the same general principles was developed. However, as usual, unique conditions peculiar to the compound under investigation were required. These are described in this communication.

MATERIALS AND METHODS

Commercial captan [*N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide] was added to various solid media at a rate of 17.7 μg. of active ingredient per gram. Ten-gram samples of these mixtures were then extracted with 20 ml. of dichloromethane and the solvent decolorized with Norite and Florisil as previously described in the analysis of 3,4-dichlorotetrahydrothiophene-1,1-dioxide (1). After evaporation of the solvent under a current of clean dry air, 10 ml. of $0.073M$ tetraethylammonium hydroxide in 70 per cent aqueous pyridine (v/v) were added to the resi-

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due. An aliquot was then transferred to a $\frac{3}{4}$ inch cell and the absorbance read at a wave length of 415 mμ using a Bausch and Lomb Spectronic 20 Colorimeter. The blank was an extract from a medium (sand or soil) that did not contain captan treated in a similar manner. Absorbances were usually measured within 2 minutes from the time the reagent was added to the residue, but the color was stable for about 30 minutes. The concentration of fungicide was interpolated from a standard curve based on data obtained on a sample of recrystallized captan (m.p. 171°–173° C.).

To minimize background, fresh reagent was prepared daily by mixing stock solutions of pyridine and tetraethylammonium hydroxide since solutions containing the two components slowly became colored on standing. Tetraethylammonium hydroxide was obtained from the Distillation Products Division of the Eastman Kodak Co. as a 10 per cent solution in water and its molarity determined by titration with standard acid. Pyridine was obtained from the Mallinckrodt Chemical Co.

The longevity of the color-forming capacity of the fungicide in $M/30$ phosphate buffer at pH 7.0 and 29° C. was determined as described previously for *s*-triazine derivatives (4, 5) and 1-fluoro-2,4-dinitrobenzene (6) except that the initial concentration of captan in the hydrolysis rate experiments was 17 μg. per ml., and 20-ml. aliquots of the buffer were extracted with 10-ml. portions of dichloromethane. Analyses for residual captan were carried out as described above.

An incinerated soil used as an experimental medium to evaluate the method for the recovery of captan was prepared by heating composted garden soil over a Meker burner until the organic matter decomposed. The pH of this material was then reduced from about 10 to its original value of 6.3 by addition of 2.4 per cent aqueous citric acid.

EXPERIMENTAL RESULTS

Attempts were made to analyze for captan by treating it consecutively with pyridine and sodium hydroxide at room temperature as was done for *s*-triazine derivatives, but the intensity of the color obtained was much too low to serve as the basis of a practical analytical method. Higher intensity was achieved when the pyridine and alkali were added in admixture as in the Fujiwara test (2), but fading was extremely rapid and Beers law was not obeyed. Color stability was greatly improved when tetraethylammonium hydroxide was substituted for sodium hydroxide. Maximum intensity was reached by the time the first absorbance reading could be made and appreciable fading did not occur within the first half hour. Color intensity was dependent on the concentrations of pyridine (Fig. 1 A) and alkali (Fig. 1 B). When pyridine concentration was held constant at an arbitrary value and the molarity of the quaternary base varied, a curve showing a maximum color intensity peak was obtained when composition was plotted against absorbance. However, when the concentration of quaternary base yielding the highest color intensity was held constant and the per cent pyridine varied, it was found that the concentration of pyridine in the initial experiment was not optimal. Similarly, when the percentage of pyridine in the reagent was fixed at the value giving the new maximum and the molarity of the base adjusted stepwise, another shift was observed in the position of the maximum. To achieve optimum concentrations of both pyridine and base it was necessary to adjust each in turn until the maxima no longer shifted. This required three experiments in which the concentration of pyridine was varied and three in which the molarity of quaternary base was changed. The reagent composition yielding maximum absorbance was found to contain 70 per cent pyridine and to be 0.073*M* in tetraethylammonium hydroxide. Under these conditions the absorption maximum was at a wave length of 415 mμ and the molar absorbance was 1.3 × 10^4 calculated on the initial concentration of captan. Beers law was followed and the coefficient of variation was found to be 5 per cent in experiments made on the same day but at different concentrations. The fungicide Phaltan [*N*-(trichloromethylthio)phthalimide] can be determined using identical con-

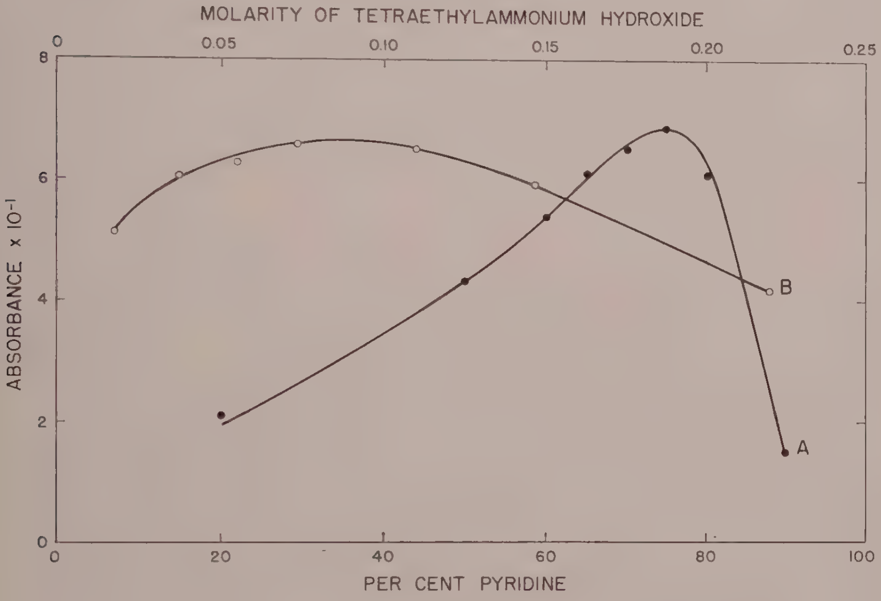

FIGURE 1. (A) Relation between color intensity and pyridine concentration in a solution containing 0.073 mole per liter tetraethylammonium hydroxide and 12.7 μg. of captan per ml. (B) Relation between absorbance and molarity of the quaternary base in a series of solutions containing 70 per cent by volume of pyridine in water and 12.7 μg. of captan per ml.

TABLE I

RECOVERY OF CAPTAN ADDED TO VARIOUS MEDIA AT A CONCENTRATION OF 17.7 μG. PER G.

Medium	Per cent recovery	Average
Dry sand	96 93 97 90	94
Moist sand	80 73 75 74 74 76	75
Incinerated soil	86 93 90	90

ditions, but the molar absorbance at 415 mμ was found to be 2.3×10^4, or about 75 per cent higher than for captan.

The method was evaluated for the analysis of captan extracted from incinerated soil, sand, and moist sand. This unusual choice of media was based on the finding of Lukens (10) that captan is chemically reactive. To avoid disappearance of the fungicide from possible reactions with organic compounds in ordinary soils, these materials were used so that recoveries would be indicative of completeness of extraction and losses through hydrolysis only. When mixed with dry sand or incinerated soil containing only enough water to permit the adjustment of pH, average recoveries were 94 and 90 per cent respectively at an initial concentration of 17.7 μg. of captan per gram (Table I). When captan was added to moist sand at the same concentration average recovery was only 75 per cent indicating that a considerable amount of the compound was lost. To determine whether this might be caused by hydrolysis, a solution containing 177 μg. of captan in acetone solution was evaporated to dryness and treated with water. On evaporation of the water under a current of air the recovery of captan by absorptiometric analysis was only 86 per cent, once more pointing to a loss by hydrolysis.

Further evidence for the rapid decomposition of captan in aqueous media was obtained by preparing a solution containing 17 μg. of the compound per ml. of *M*/30 phosphate buffer at pH 7. At various time intervals aliquots of the buffer were extracted with dichloromethane and the organic phases evaporated to dryness. Absorptiometric analyses showed that color intensity was reduced to one-half of its initial value within 2.5 hours at 29° C. (Fig. 2).

Evidently captan and/or an intermediate color-producing agent such

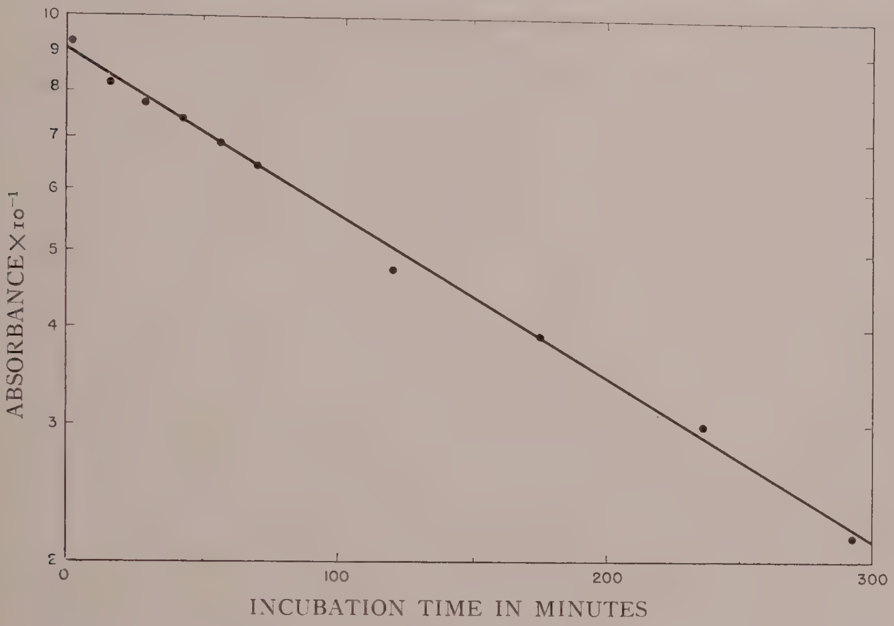

FIGURE 2. Relation between absorbance (on a logarithmic scale) and incubation time for a solution containing an initial concentration of 17 µg. of captan per ml. of *M*/30 phosphate buffer at pH 7 and 29° C.

as the thiophosgene postulated by Lukens and Sisler (8, 9, 10) decomposes very rapidly in this medium. It is not known whether this latter compound yields a chromophore on treatment with pyridine-alkali reagent.

DISCUSSION

This method for the analysis of captan probably depends on the ability of the halogens of its trichloromethyl group to react with pyridine and the quaternary base according to the general scheme previously proposed for a number of other fungicides and related compounds (2). However, the reaction mechanisms are probably much more complex with these compounds owing to the presence of 3 reactive chlorine atoms attached to the same carbon (9), and the probable instability of the initial reaction products. This is suggested by the fact that Lukens (8) and Lukens and Sisler (10) found the reaction products of captan and cysteine to be predominantly tetrahydrophthalimide, cystine, hydrogen sulfide, carbon disulfide and 2-thiazolidinethione-4-carboxylic acid, indicating that the products formed on displacement of the halogens are highly unstable and capable of undergoing a variety of secondary reactions. This view is supported by the low color intensities obtained in the analytical procedure when the pyridine and base were added consecutively rather than simultaneously. Presumably, stepwise addition allowed sufficient time for the intermediates to react further to yield products which did not generate strong chromophores in the presence of bases. Another point of interest in this procedure is the finding that color stability was much better in the presence of tetraethylammonium hydroxide than when sodium hydroxide was used. This is not without precedent since benzyltrimethylammonium hydroxide gave better stability than sodium hydroxide in the analysis of the dichloro-*s*-triazines (3). However, in this latter case either reagent could be employed while in the analysis of captan the use of the organic base was essential.

Captan was much less stable in aqueous buffer than the dichloro-*s*-triazines (4) or 1-fluoro-2,4-dinitrobenzene (6). The time required for the intensity of the color produced in the analytical test to diminish by one-half was only about 2.5 hours compared to 20 to 30 days for the former compounds. The relation between the logarithm of absorbance and incubation time was linear (Fig. 2) as would be expected in a first order reaction and as has been found for the other fungicides referred to above. This suggests that captan hydrolyzes in water directly to materials which do not form chromophores with pyridine-base, for otherwise aberrations would appear in the rate curves.

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INFLUENCE OF pH AND GROUP INTERACTIONS ON THE REACTIVITIES OF METABOLITES AND RELATED COMPOUNDS CONTAINING SULFHYDRYL GROUPS

H. P. Burchfield and Eleanor E. Storrs[1]

SUMMARY

The apparent reactivities of metabolites and related compounds containing sulfhydryl groups with the fungicides 1-fluoro-2,4-dinitrobenzene (FDNB) and 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine (Dyrene) at pH 6 and 29° C. decreased in the order: cysteine ethyl ester > mercaptoethylamine > glutathione = cysteine > homocysteine. Cysteine ethyl ester was about 8 times as reactive as homocysteine with Dyrene and over 12 times as reactive with FDNB. The apparent reactivities of cysteine and glutathione with either fungicide were the same within experimental error, but both metabolites reacted at slightly higher rates with Dyrene. On changing pH from 6 to 7 a 10-fold increase was found in the apparent reaction rates of cysteine, homocysteine, and glutathione. Although similar effects were observed for cysteine ethyl ester and mercaptoethylamine, aberrations in the rate curves at the higher pH prevented quantitative comparisons being made.

Taking into account the ionization of the sulfhydryl groups, the velocity coefficients for the reactions of the $^{-}SRNH_3^+$ ions with FDNB decreased in the order: homocysteine > glutathione > mercaptoethylamine ≧ cysteine ≧ cysteine ethyl ester. The intrinsic reactivity of the $^{-}SRNH_3^+$ ion of cysteine was only about one-fifth that of the corresponding ion of glutathione, presumably because of the electron-attracting capacity of the NH_3^+ group adjacent to the sulfhydryl. At pH values where the $^{-}SRNH_2$ ion predominates, cysteine should be the more reactive.

The reactions obeyed first order but probably bimolecular kinetics with respect to the disappearance of the fungicides in the presence of 10 to 100 molar equivalents of the sulfhydryl compounds. However, when the rate of reaction of cysteine with FDNB was followed by measuring light absorption by the final reaction product at 360 mμ, first order kinetics were not strictly obeyed, and the apparent rate of formation of the end product was much slower than the rate of disappearance of the fungicide. Subsequent investigation showed that this was probably caused by an initial reaction at the RS^- group followed by rearrangement to *N*-(2,4-dinitrophenyl)cysteine. Spectrophotometric evidence indicates that this occurs with cysteine and β-mercaptoethylamine, but probably not with homocysteine and glutathione.

INTRODUCTION

Previous investigations (14) of the reaction rates of amino acids and peptides with the fungicide 2,4-dichloro-6-(*o*-chloroanilino)-*s*-triazine (hereafter referred to as Dyrene) demonstrated that cysteine and glutathione had about the same apparent reactivities before ionization of the functional groups were taken into consideration. This result did not agree with the views of Calvin (17) who argued that the reduced form of glutathione is more stable because it exists in equilibrium with a derivative of

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[1] The authors wish to thank William R. Smith for the infrared determinations and Joan Schechtman for technical assistance in part of this work.

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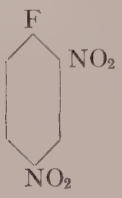
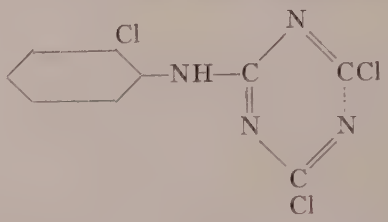
thiazoline, the investigations of Benesch *et al.* (5) who believed cysteine to be the more reactive because it gave higher color intensity in the nitroprusside test, or the practical experience of many workers who have reported that glutathione is less susceptible to oxidation. In view of these apparent discrepancies, additional studies of the reaction rates of Dyrene and 1-fluoro-2,4-dinitrobenzene (FDNB) with homocysteine, cysteine, cysteine ethyl ester, glutathione, and β-mercaptoethylamine were made.



FDNB



Dyrene

The experimental results and some approximate values for the reactivities of the $^{-}SRNH_3^{+}$ ions are given together with a discussion of the toxicological and physiological significance of variations in inter- and intracellular pH values and the interactions of functional groups in metabolites.

MATERIALS AND METHODS

Glycine, DL-α-alanine, glutathione, DL-homocysteine, and L-cysteine ethyl ester hydrochloride were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, and β-mercaptoethylamine from the California Foundation for Biochemical Research, Los Angeles, Calif. Samples of L-cysteine purchased from the Nutritional Biochemicals Corp., the Distillation Products Division of the Eastman Kodak Co., Rochester, N. Y., and Mann Research Laboratories Inc., New York, N. Y. gave comparable results in kinetic studies. FDNB was supplied by the Aldrich Chemical Co., Milwaukee, Wis., while the preparation and properties of Dyrene (formerly designated as experimental compound B-622) have been described elsewhere (13, 14). The sulfhydryl contents of the metabolites were checked by iodometric titration. Satisfactory results were obtained except for cysteine, which is known to oxidize past the disulfide stage in amounts that are concentration-dependent (41).

Kinetic studies were carried out at 29° C. in aqueous $M/30$ phosphate buffer containing 1 per cent acetone. The initial concentration of Dyrene or FDNB was 5 μg. per ml. (1.8×10^{-5} and 2.7×10^{-5} moles per liter, respectively) while 15 to 200 molar equivalents of sulfhydryl compounds were used at pH 6, and 5 to 20 equivalents at pH 7. Each experiment was repeated 4 to 6 times, usually using different concentrations of the thiols. For convenience, average $t_{1/2}$ values were calculated on the basis of 100 equivalents of metabolite at pH 6, and 10 equivalents at pH 7, since the concentration-time relationship was found to be quantitative within experimental error.

Reactions were stopped at various time intervals by adding 1 ml. of $2N$ aqueous silver nitrate to 10 ml. of the reaction mixtures to precipitate both the sulfhydryl compounds and the buffer. The aliquots were then quickly extracted with 10 ml. of dichloromethane, and a 5-ml. aliquot of the latter solvent evaporated to dryness. The amounts of Dyrene or FDNB in the residues were determined by methods described elsewhere (12, 13, 14, 15).

The reactions of cysteine and glycine with FDNB were also investigated by measuring the increases in light absorption at 360 mμ resulting from the formation of their dinitrophenylated products. Reaction conditions were the same as described above except that measurements were made in 1-cm. Corex cells using a Beckman Model DU spectrophotometer. The change in $\lambda_{max.}$ during the reaction of cysteine with FDNB was measured on a Perkin-Elmer Model 13 Spectrophotometer at pH 7 and 23° to 26° C., the initial concentrations of FDNB and cysteine being 5.4×10^{-5} moles per liter and 5.4×10^{-4} moles per liter respectively.

In view of the pH dependence of these reactions (14, 15), solutions were adjusted to the desired values using a model GS pH meter with a sensitivity of 0.0025 pH unit. The instrument was standardized against $0.025M$ potassium phosphate buffer supplied by the Beckman Instrument Co. These buffers are checked with National Bureau of Standards certified buffer solutions and held to rigid specifications. The pH of the buffer was within 0.02 unit of 6.98 at 29° C. The instrument can detect differences smaller than this, so new buffers were standardized against the original one. This introduced a systematic error about equal to the amount the initial buffer deviated from neutrality. However, pH measurements are based on arbitrary standards (42, 46) and in this present work reproducibility was considered more important than absolute accuracy. Careful selection of glass and calomel electrodes was essential in measuring pH with this precision even when the instrument was used for null point readings at pH 7.

The reaction product of FDNB and cysteine was prepared by dissolving cysteine hydrochloride (15.7 g.) in 1 liter of boiled distilled water and adjusting the pH to 8 with Na_2HPO_4. FDNB (1.86 g.) was added and the solution warmed on a hot plate until a positive pyridine-alkali test was no longer obtained. The solution was then acidified with HCl and extracted with ether. The ether layer was washed with water and next extracted with $0.1N$ NaOH to remove the reaction product. A few drops of

$FeCl_3$ were added to the aqueous phase, and air bubbled through it until a positive nitroprusside test was no longer obtained. The reaction product was precipitated by acidifying with HCl and collected on a Buchner funnel. It was dissolved in hot ethylene glycol monomethyl ether, filtered and quenched with water. It was then recrystallized from acetic acid-water. Its infrared spectrum was compared with an authentic sample of *N,N′*-bis-(2,4-dinitrophenyl)cystine (Fig. 1) obtained from the Mann Research Laboratories, New York, N. Y. Neither this product nor the authentic sample had sharp melting points.

NATURE OF THE REACTIONS

The mechanisms of the reactions of FDNB and Dyrene with thiols have not been studied extensively, but some information is available on the reactions of iodoacetate and halonitrobenzenes with aliphatic and aromatic amines. Brdička (8) found that the reaction that takes place between iodoacetate and glycine is pH dependent, and suggested that only the ionized amino group ($R\text{-}NH_2$) can react as has been proposed here (14, 15). Bunnett and Pruitt (9), during a study of the reactions of piperidine with 2,4-dinitrobenzenes substituted in the 1-position with halogens, phenolic and sulfone groups, found that sodium hydroxide did not increase reaction velocities and suggested that such reactions were not base-catalyzed. This is in agreement with present evidence which suggests that the only effect of increasing alkalinity is to make available greater concentrations of the reactive $^{-}SRNH_3^{+}$ and $^{-}SRNH_2$ ions. Chapman *et al.* (20, 21) have studied the reactions of FDNB and found that it combines more rapidly with amines than its chlorine analogue. More recently Ross and Finklestein (38) studied the action of 1-chloro-2,4-dinitrobenzene on a number of amines and concluded that within the limitations of their measurements the reactions were truly bimolecular. Thus there is strong evidence that the reactions of some active halogen compounds with amines, and by inference sulfhydryl compounds (33, 43), are bimolecular and of the S_{N_2} type, and that their pH dependence arises solely from differences in the degrees of ionization of the functional groups. There are, however, certain classes of halogeno compounds which react with RNH_2 and RS^- by an S_{N_1} mechanism, the rate depending on ease of ionization of halogen to give a carbonium ion (36, 39, 40).

In the present studies the reaction between FDNB and cysteine was studied at a constant initial concentration of fungicide and 15 to 200 molar equivalents of the thiol. The reaction rate, as measured by the disappearance of the FDNB, obeyed first-order kinetics at pH 6. The half-times of the reactions varied from 21 to 268 minutes (Table I), but the apparent bimolecular velocity coefficient (calculated on the total initial concentration of cysteine) was constant to within 10 per cent. The reactions of homo-

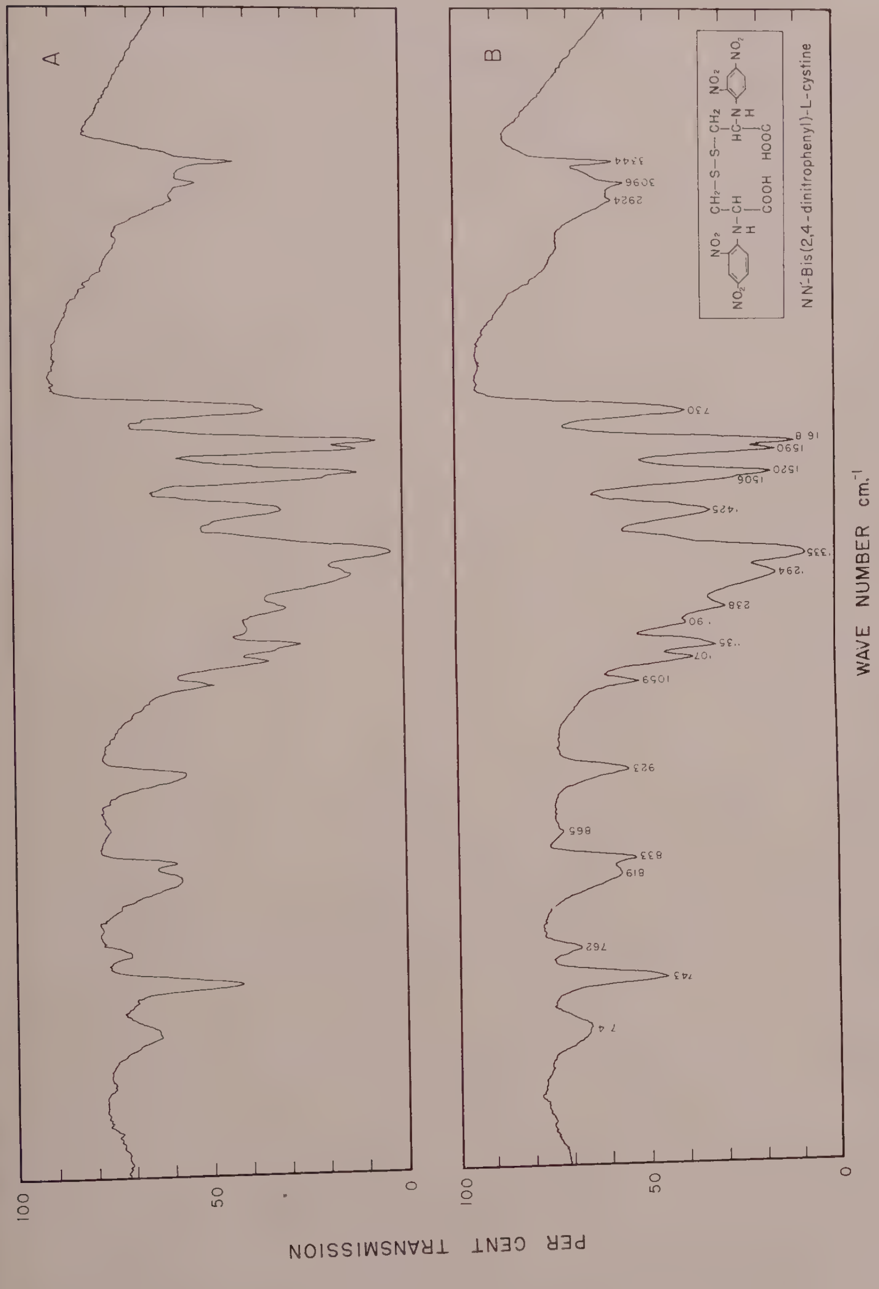


FIGURE 1. Infrared absorption spectra of the reaction product of FDNB and cysteine after oxidation (A) and an authentic sample of *N*,*N*′-bis(2,4-dinitrophenyl)cystine (B).

TABLE I

APPARENT RATE CONSTANTS FOR THE REACTION OF FDNB WITH VARIOUS CONCENTRATIONS OF CYSTEINE AT pH 6.0

Moles cysteine per mole FDNB	$t_{1/2}$ (Minutes)	k (Mole liter^{-1} sec.$^{-1}$)
15	268	0.11
100	39	0.11
200	21	0.10

cysteine and glutathione with both FDNB and Dyrene also obeyed first order kinetics at pH values of 6 and 7. In the case of cysteine a small decrease in the slope of the cumulative rate curve (Fig. 2) occurred in the vicinity of the half-time at pH 7.0 but not at pH 6.0. Evidently this did not arise from inhibition of the reactions by their products since neither the addition of fluoride or chloride ions nor the reaction products of cysteine with FDNB and Dyrene produced any further change. To avoid oxidation of sulfhydryl groups kinetic experiments were made under an atmosphere of tank nitrogen. Oxygen tension was further reduced by passing the nitrogen through a heated combustion tube containing copper turnings, but without any improvement in results. Finally, a sealed ap-

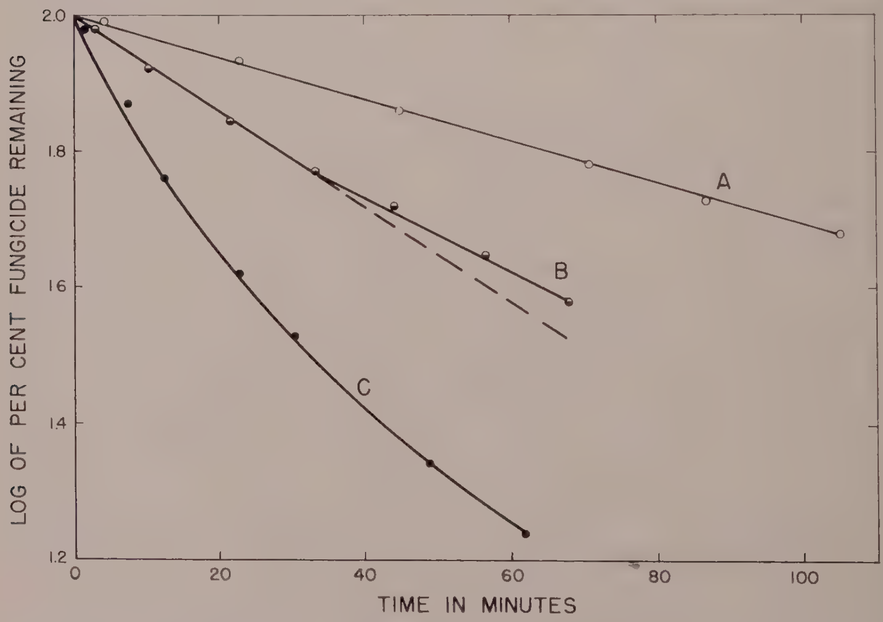


FIGURE 2. Reaction rates of fungicides at initial concentrations of 5 μg. of FDNB or Dyrene per ml. and 10 molar equivalents of sulfhydryl compound at pH 7. (A) Dyrene with homocysteine. (B) FDNB with cysteine. (C) Dyrene with cysteine ethyl ester.

paratus was constructed in which all solutions were prepared and transferred under oxygen-free nitrogen, but aberrations in the rate curves still appeared. These were not large in the case of cysteine, but were much more pronounced in the reactions of both β-mercaptoethylamine and cysteine ethyl ester with FDNB and Dyrene (Fig. 2). When the pH was reduced to 6 and the concentration of thiol increased to 50 to 100 molar equivalents, the aberrations disappeared almost entirely (Fig. 3), presumably because the solutions were more highly poised by the increased concentrations of the sulfhydryl compounds. Aberrations were not observed at either pH with glutathione, so rate studies were carried out at pH 8 using 5.4×10^{-5} moles per liter of glutathione and 2.7×10^{-5} moles per liter of FDNB to determine the molecularity of the reaction. As expected, the relationship between time and the logarithm of light absorption was not linear, but on transforming the data to second order parameters (Fig. 4), a linear relationship was obtained until 70 per cent of the FDNB had reacted, indicating that the reaction is probably truly bimolecular within the limits of error of these measurements.

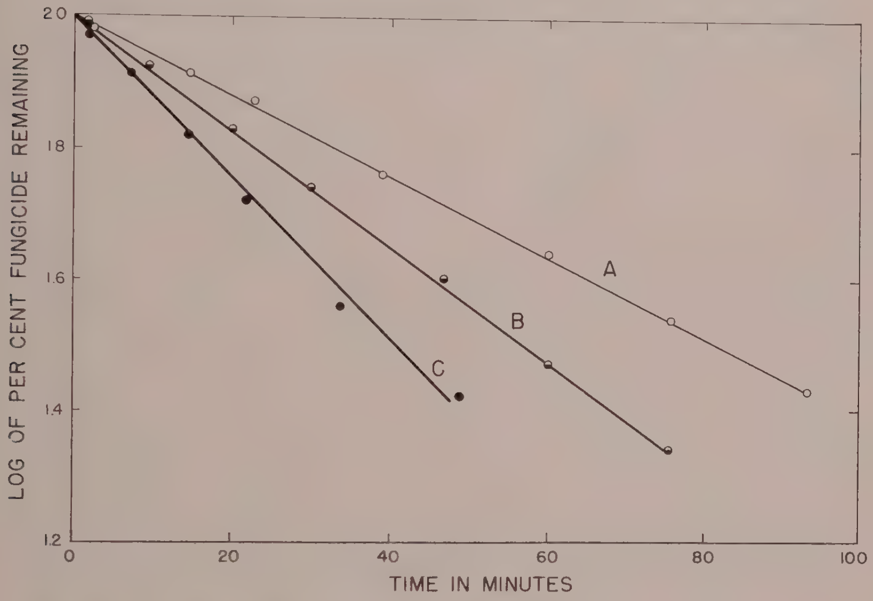


FIGURE 3. Reaction rates of fungicides at pH 6 at initial concentrations of 5 μg. per ml. (A) Dyrene in the presence of 100 molar equivalents of glutathione. (B) FDNB in the presence of 50 molar equivalents of β-mercaptoethylamine. (C) Dyrene in the presence of 50 molar equivalents of cysteine ethyl ester.

Even though the reaction of cysteine with FDNB is probably also bimolecular and thus dependent on the concentration of $^{-}SRNH_3^{+}$ ion, complications occurred when attempts were made to follow the reaction by measuring the light absorption of the final product at 360 mμ. The time required for one-half of the final product to appear was about 65 per cent longer than for one-half of the FDNB to be used up, indicating a block en route (Fig. 5). By contrast the reaction of FDNB with glycine proceeded normally, the two half-times being equivalent to within the limits of error of the experiments. The absorption maximum of the reaction product of FDNB and cysteine was very close to the value obtained for *N*-(2,4-dinitrophenyl)glycine and about 20 mμ higher than that for the reaction product of FDNB and mercaptoacetic acid. It would seem from these results that *N*-(2,4-dinitrophenyl)cysteine was the final product. However, the reaction rate was about one thousand times faster than usually found for RNH_2, suggesting that the primary reaction was with the RS^{-} group. Measurements made with a recording spectrophotometer showed that the net absorption maximum of the products changed from about 340 to 345 mμ at the beginning of the reaction to 361 mμ near its completion (Fig. 6). Further evidence for the initial formation of an *S*-substituted intermediate was obtained in an experiment in which it was pos-

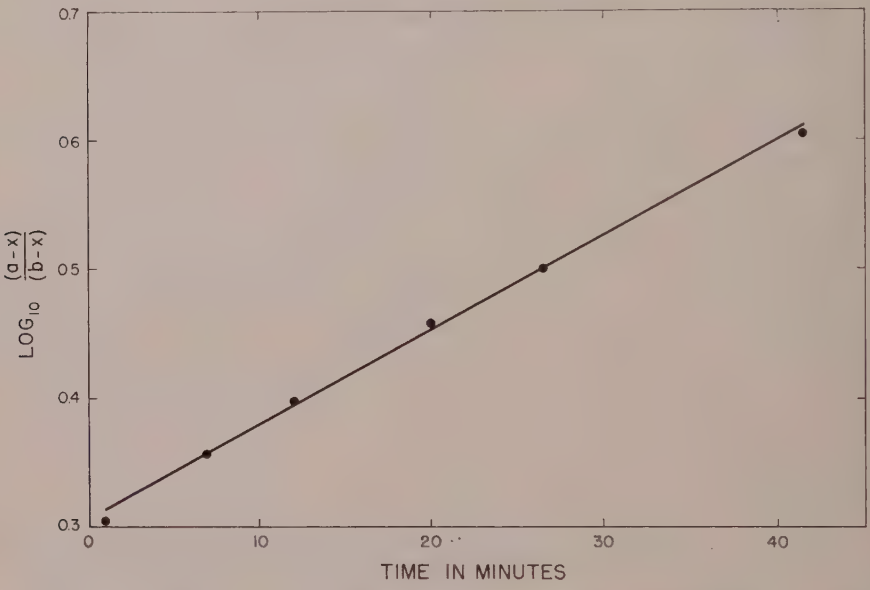


FIGURE 4. Second order plot for the reaction of FDNB with glutathione at pH 8 where *a* is the initial molarity of glutathione, *b* the initial molarity of FDNB, and *x* the moles of FDNB reacted at time *t*.

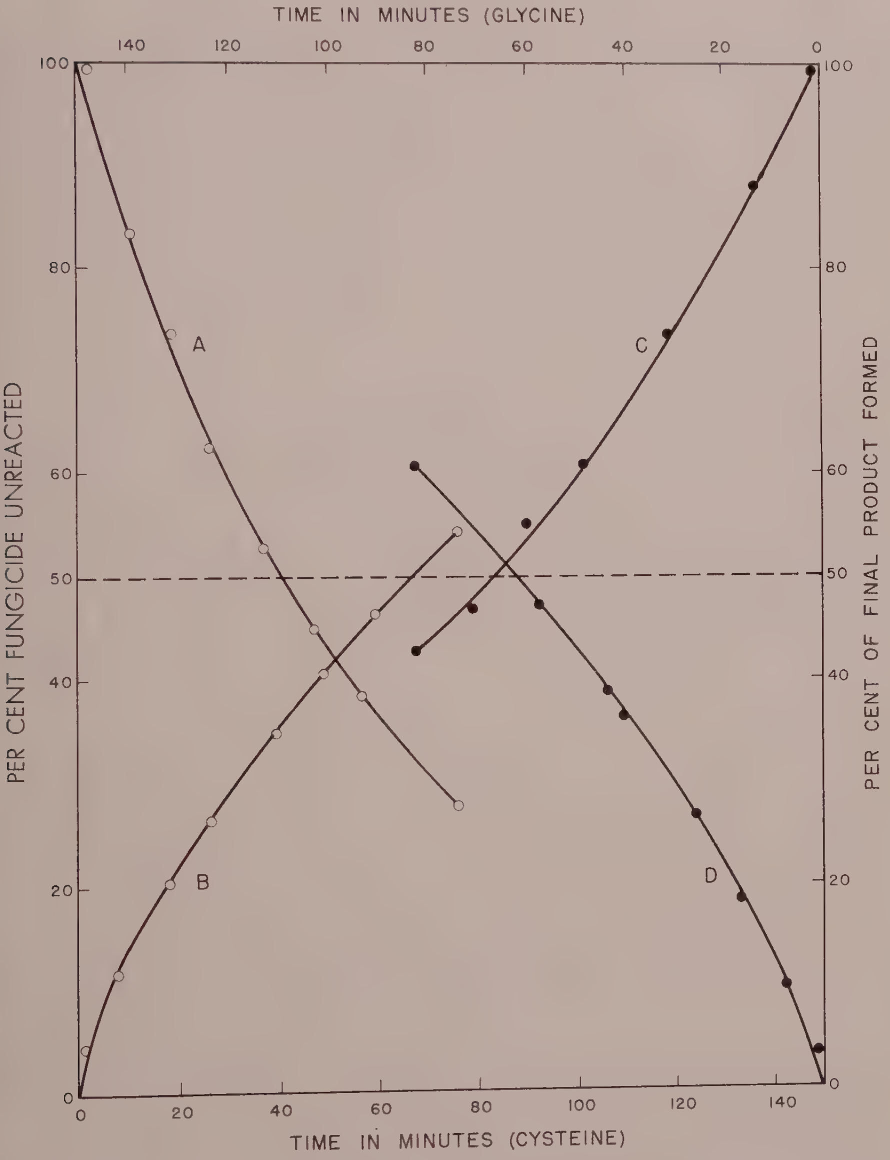


FIGURE 5. Reactions of FDNB with cysteine at pH 7, and with glycine at pH 8. (A) Relation between time and amount of FDNB reacted in the presence of 10 molar equivalents of cysteine. (B) Formation of the reaction product of FDNB and cysteine measured by optical readings at 360 mμ. (C) Relation between time and amount of FDNB reacted in the presence of 10^3 molar equivalents of glycine. (D) Formation of *N*-(2,4-dinitrophenyl)glycine as determined from optical measurements at 360 mμ.

sible to separate it from the final product by solvent extraction mid-way through the reaction (11). The end-product contained a free sulfhydryl group, as in *N*-(2,4-dinitrophenyl)cysteine, since when isolated from the reaction mixture it gave a positive nitroprusside test, and reduced iodine. The infrared spectrum also supported the view that substitution had occurred on the nitrogen atom. The band at 2000 cm.$^{-1}$ characteristic for amino acids containing the NH_3^+ group, but seldom occurring in secondary amines (3, p. 200), was not found. A weak band at 2577 cm.$^{-1}$ was well within the range of 2550 to 2600 cm.$^{-1}$ where SH absorption usually occurs (3, p. 288). On oxidizing the material with air in the presence of $FeCl_3$ a product was formed which had the same infrared absorption spectrum as an authentic sample of *N*,*N'*-bis(2,4-dinitrophenyl)cystine (Fig. 1).

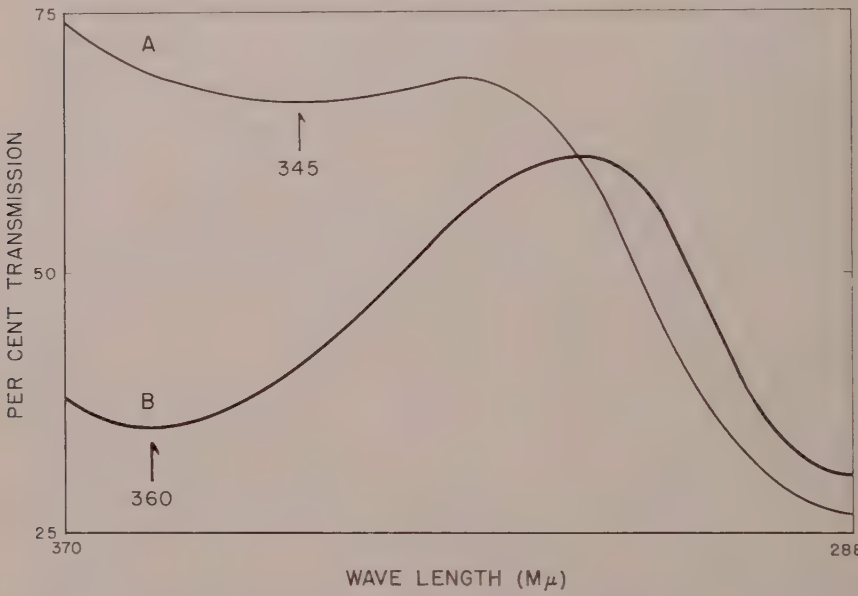


FIGURE 6. Ultraviolet absorption spectra of the reaction products of FDNB with cysteine at pH 7. (A) Fifteen minutes after the beginning of the reaction. (B) One and three-fourths hours later.

This rearrangement may occur with compounds other than cysteine since β-mercaptoethylamine when caused to react with FDNB formed a product with an absorption maximum at 361 mμ (Table II). However, the absorption maximum obtained with cysteine ethyl ester was intermediate between those of the glycine and mercaptoacetic acid reaction products, so it is not certain whether a rearrangement had taken place. The reaction products of FDNB with homocysteine and glutathione had absorption maxima in the same range as observed for *S*-(2,4-dinitrophenyl)mercapto-

TABLE II

WAVELENGTHS OF MAXIMUM ABSORBANCE FOR FINAL REACTION PRODUCTS OF FDNB WITH VARIOUS COMPOUNDS AT pH 7.0

Reactant	$\lambda_{max.}$ of product (mμ)
Mercaptoacetic acid	330
DL-Homocysteine	341
Glutathione	338
Glycine	361
DL-α-Alanine	361
L-Cysteine	361
L-Cysteine ethyl ester	346
β-Mercaptoethylamine	361

acetic acid, so it seems probable that rapid molecular rearrangements do not take place under these conditions unless the sulfhydryl and amino groups are in close proximity to one another. Reactions similar to this can take place at higher temperature and pH values even when the groups are on different molecules. Thus Zahn and Kockläuner found that *O*-(2,4-dinitrophenyl)tyrosine reacts with glycine at pH 9.2 when heated to 87° C., yielding *N*-(2,4-dinitrophenyl)glycine (48, 49), while later Zahn and Pfannmüller (50) showed that the aromatic group attached to the heterocyclic ring of *N-imid.*-bis(2,4-dinitrophenyl)-L-histidine migrated to the amino group of glycine under similar conditions.

The reactions between cysteine and FDNB are therefore complex since theoretically they can take place in the following ways.

$$(1)\quad {}^{-}SRNH_3^{+} + FDNB \xrightarrow{k_1} (NO_2)_2\phi SRNH_3^{+}$$

$$(2)\quad {}^{-}SRNH_2 + FDNB \xrightarrow{k_2} (NO_2)_2\phi SRNH_2$$

$$(3)\quad {}^{-}SRNH_2 + FDNB \xrightarrow{k_3} {}^{-}SRNH\phi(NO_2)_2$$

$$(4)\quad HSRNH_2 + FDNB \xrightarrow{k_4} HSRNH\phi(NO_2)_2$$

$$(5)\quad (NO_2)_2\phi SR\text{-}NH_3^{+} \xrightarrow{k_5} HSRNH\phi(NO_2)_2$$

$$(6)\quad {}^{-}SRNH\phi(NO_2)_2 + FDNB \xrightarrow{k_6} (NO_2)_2\phi SRNH\phi(NO_2)_2$$

In addition, ionic species with undissociated carboxyl groups can participate, and there is some evidence that oxidation and other side reactions occur. Fortunately most of these reactions are slow or can be suppressed if the appropriate reaction conditions are chosen, so it is possible to compare the reactivities of the compounds on the assumption that equation (*1*) represents the predominant pathway through which FDNB and Dyrene are removed from the system.

RELATIVE REACTIVITIES

The experimental data for the reactions of Dyrene and FDNB with the five compounds are given in Table III so that rate constants can be recalculated should improved methods become available. Data for cysteine ethyl ester and β-mercaptoethylamine at pH 7 were not included since the values are not considered reliable. Otherwise the values of $t_{1/2}$ are accurate to within about 1 per cent in a single experiment or to within 5 per cent in independent experiments (15).

TABLE III

AVERAGE HALF-TIME VALUES FOR THE DISAPPEARANCE OF DYRENE AND FDNB IN THE PRESENCE OF 100 MOLAR EQUIVALENTS OF SULFHYDRYL COMPOUND AT pH 6.0 AND 10 EQUIVALENTS AT pH 7.0

Compound	$t_{1/2}$ (Minutes)			
	Dyrene		FDNB	
	pH 6.0	pH 7.0	pH 6.0	pH 7.0
L-Cysteine ethyl ester	13	—	6	—
β-Mercaptoethylamine	19	—	17	—
L-Cysteine	45	50	40	39
Glutathione	47	51	36	38
DL-Homocysteine	104	96	78	72

Apparent second-order velocity coefficients for the reactions of FDNB with the various thiols were calculated on the erroneous but useful assumption (15) that all ionic species participated equally (Table IV). The rate constants varied from a low of 0.056 mole liter^{-1} sec.$^{-1}$ for homocysteine at pH 6 to 0.70 mole liter $^{-1}$ sec.$^{-1}$ for cysteine ethyl ester, or by a factor of 12, so evidently the location of a group within a microenvironment (10) affects its reactivity considerably. Comparable results were obtained with Dyrene (Table IV). These two fungicides parallel one another with respect to their reactivities with this group of substances, but correlation is not

TABLE IV

APPARENT REACTIVITIES OF FDNB AND DYRENE WITH SULFHYDRYL COMPOUNDS AT 29° C.

Compound	k (Mole liter^{-1} sec.$^{-1}$) at pH 6.0		k (Mole liter^{-1} sec.$^{-1}$) at pH 7.0		k_7/k_6 (Average)
	FDNB	Dyrene	FDNB	Dyrene	
DL-Homocysteine	0.056	0.062	0.62	0.68	11
L-Cysteine	0.11	0.14	1.1	1.3	9.7
Glutathione	0.12	0.14	1.2	1.3	9.7
β-Mercaptoethylamine	0.25	0.35	—	—	—
L-Cysteine ethyl ester	0.70	0.50	—	—	—

quantitative, even when experimental errors are considered. For example, FDNB is about 40 per cent more reactive with cysteine ethyl ester than is Dyrene, but about 30 per cent less reactive than is Dyrene with β-mercaptoethylamine.

The apparent reactivities of these sulfhydryl-containing compounds with both FDNB and Dyrene decreased in the order: cysteine ethyl ester $>\beta$-mercaptoethylamine $>$ glutathione $=$ cysteine $>$ homocysteine. The apparent reactivities of homocysteine, cysteine, and glutathione with both FDNB and Dyrene increased 10-fold (k_7/k_6 in Table IV) when the pH was increased from 6 to 7, illustrating once more that ability to ionize is the principal factor in this phenomenon.[2] The observation that cysteine and glutathione reacted at the same rates regardless of whether the fungicide was FDNB or Dyrene was of considerable interest. As noted in the introduction, this finding is at variance with the views of some other workers that glutathione is the more stable compound, particularly with respect to ease of oxidation. It was at first thought that this discrepancy could be explained by assuming that glutathione was ionized to a greater extent than cysteine under the experimental conditions, in agreement with the older assignment by Cohn and Edsall (22, p. 85) of the pK′ values of cysteine rather than the more recent assignment proposed by other authors (4, 17). Thus the pK′ values of the sulfhydryl and amino groups of cysteine were formerly considered to be 10.28 and 8.18 respectively, compared with a value of 9.12 for the sulfhydryl group of glutathione (22, p. 85). According to these values, the amount of RS^- available for reaction would be one hundred times higher for glutathione than for cysteine, thus inferring that cysteine had the higher intrinsic reactivity, but that this was suppressed by its weaker acidity. The idea that cysteine had the higher reactivity was derived from the work of Benesch *et al.* (5) who based their conclusion on the finding that it gave a higher optical absorbance than glutathione in the nitroprusside test. Since this reaction is carried out in alkaline solution (Na_2CO_3-$NaCN$) where both compounds are highly ionized, it should be a measure of intrinsic rather than apparent reactivity, providing, of course, that it is possible to relate color intensity to reactivity. However, it was pointed out to the authors[3] that a spectrophotometric method had been used for the measurement of RS^- ions which appeared to make the assignment of pK values unequivocable. Using this method, Benesch and Benesch (4) described the ionization of cysteine as follows, neglecting the carboxyl groups which are almost completely dissociated at pH 7.

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[2] The 10-fold increase in reactivity per unit pH is an approximation and is valid only when the upper pH value is one unit or more lower than the pK of the ionizing group.

[3] Benesch, Reinhold. Personal communication.

$$\begin{tikzcd}
\text{I}\quad HSRNH_3^+ \arrow[r, shift left, "K_A"] \arrow[d, shift right, "K_B"'] & {}^-SRNH_3^+\quad \text{II} \arrow[l, shift left] \arrow[d, shift left, "K_C"] \\
\text{III}\quad HSRNH_2 \arrow[u, shift right] \arrow[r, shift left] & {}^-SRNH_2\quad \text{IV} \arrow[l, shift left, "K_D"] \arrow[u, shift left]
\end{tikzcd}$$

The values for pK_A, pK_B, pK_C, and pK_D were found to be 8.53, 8.86, 10.36, and 10.03 respectively, showing that the sulfhydryl group of cysteine is more highly ionized at equivalent pH than in glutathione, since the pH at which the concentration of RS^- equals the concentration of RSH is 9.20 for this latter compound. De Deken *et al.* (27) using the same technique arrived at opposite conclusions. They accepted the older assignment of pK values with slight modifications and suggested that the amino group was first to lose a proton, and that it then became associated with the SH group through hydrogen bonding. However, if it is conceded that the ionic species of glutathione predominating at pH 7 is more reactive than the corresponding cysteine ion, the kinetic data tend to support the results of Benesch and Benesch (4). They reported the amount of RS^- at pH 7.4 in solutions of cysteine ethyl ester, β-mercaptoethylamine, and cysteine to be 16, 11, and 6 per cent of the total, respectively. These values decrease in the same order observed for the apparent reactivities of these compounds with FDNB and Dyrene (Table IV) suggesting that ability of the sulfhydryl group to ionize is a salient factor in governing reactivity.

Calculation of accurate values for the intrinsic reactivities of these compounds is not possible at present because of uncertainties in the values of temperature coefficients, activity coefficients, etc. Nevertheless approximate values were obtained by adjusting pK values to compensate for the temperature difference in the kinetic and ionization experiments, and by regarding pH values at which $[RSH]=[RS^-]$ as equivalent to pK values (Table V).

With the exception of cysteine ethyl ester, the predominant *reactive* ion at pH 6 is ${}^-SRNH_3^+$ (II). In some cases III is present in comparable amounts but the reactivity of the RNH_2 group towards Dyrene and FDNB is only about one-hundredth to one-thousandth that of the RS^- group, so its contribution can be neglected. The ionic species IV is probably highly reactive, but its concentration is very low at pH 6. These considerations do not apply to cysteine ethyl ester, so the reactivity of II is between the limits stated assuming that IV is between 1 and 15 times as reactive as II.

Thus the reactivities of the ${}^-SRNH_3^+$ ions with FDNB decrease in the order: homocysteine > glutathione > mercaptoethylamine ≧ cysteine ≧ cysteine ethyl ester. It is now clear that the reactivity of the ${}^-SRNH_3^+$ ion of glutathione with these fungicides is about five times higher than for

TABLE V

APPROXIMATE VELOCITY COEFFICIENTS FOR THE REACTIONS OF VARIOUS $^{-}SRNH_3^{+}$ IONS WITH FDNB AT 29° C. CALCULATED FROM DATA AT pH 6.0

Compound	k' (Mole liter^{-1} sec.$^{-1}$)
L-Cysteine ethyl ester	10–20
L-Cysteine	30
β-Mercaptoethylamine	45
Glutathione	150*
DL-Homocysteine	440*

* Assuming pH at which $[SH]=[S^-]\doteq pK$.

the corresponding ion of cysteine, and that the apparent reactivities of the two compounds are about the same at pH 6 because of differences in pK. The comparatively low reactivity of cysteine in this instance must be ascribed to the electron-attracting capacity of the NH_3^+ group on the carbon atom adjacent to the RS^- group making it less susceptible to attack by electrophilic reagents. In the case of glutathione the amino group is involved in peptide bond formation and is no longer charged. This more than counterbalances steric hindrance. It is interesting to note that the $^{-}SRNH_3^{+}$ ion of homocysteine, in which the carbon atoms bearing the two functional groups are separated by a methylene group is more reactive than either cysteine or glutathione. This is further evidence that the present treatment is correct in principle since intervening methylene groups are known to dampen similar electrical interactions. The $^{-}SRNH_3^{+}$ ion of cysteine is less reactive than the corresponding ion of glutathione, but this would probably not be true of the $^{-}SRNH_2$ ion which predominates in more alkaline media, so the reactivity of cysteine (IV) would be as high or higher than for homocysteine and glutathione. Thus ionization of both amino and sulfhydryl groups probably increases the reactivity of cysteine, while ionization of the amino group may be much less important in the case of glutathione owing to the greater distance which separates it from the sulfhydryl group. It is evident that in any consideration of the reactivities of amino acids and peptides it is necessary to be specific about the ionic species involved, since in some respects they can be regarded as interrelated but different compounds.

PHYSIOLOGICAL pH

The degree of ionization of the functional groups of amino acids, peptides, and proteins will probably determine to a significant extent their reactivity towards electrophilic reagents and will hence contribute to the pharmacological effects which a particular drug will manifest. Support for this view is forthcoming from the fact that the ionization constants of amino and sulfhydryl groups differ by more than 4 orders of magnitude depending on the microenvironments (10) in which they exist. Thus the pK′ of ethylamine is 10.67, that of the amino group of asparagine 8.80, and recently Yon (47) has shown that the pK′ values of the α-amino groups liberated by the enzymatic hydrolysis of β-lactoglobulin lie between 6.4 and 7.4. Similarly the pK values of sulfhydryl groups vary from 7.45 (pK_A) for cysteine ethyl ester to 10.34 for mercaptoacetic acid (4). The hydroxyl groups of serine and threonine have not been shown to undergo interactions with other groups resulting in increased ionization but it is possible that the attachment of the proton to the oxygen can be weakened by hydrogen bonding (23) with groups in adjacent peptide chains.

Although pK values have been shown to vary considerably, it is sometimes assumed that "physiological" pH is close to neutrality because the pH of human blood plasma is 7.4, and some workers (18, 19) maintain that the cytoplasmic pH of all living cells is within 0.2 unit of 6.8 at all times. However, Wiercinski (46, p. 48) points out that "this can scarcely be true unless one completely disregards the results of all other investigators." In his review on this subject he tabulates results obtained on different Phyla of the Animal Kingdom. Most of these values lie between 5 and 8 but there are a number of exceptions. The pH of the food vacuole of *Paramecium* sp. is listed as 1.0 while at the other extreme the pH range of the cytoplasm of mature *Sabellaria alveolata* is given as 7.8 to 12. Small (42, p. 28) points out the difficulties of obtaining reliable pH values on plant cells, and states that measurements made on the sap expressed from plants have "somewhat the same significance as would determinations of the pH of an average chemical laboratory by a process which involved as its first step the crushing and mixing of all the receptacles and contents." The application of nontoxic acid-base indicators to living cells probably gives more reliable results, but even so there is considerable doubt whether they give true pictures of the pH values within metabolizing cells. There is reason to believe that cells are not uniform throughout, with respect to pH, and that there is considerable variation between species. Miller (34, p. 45) points out that the usual pH range of plant cell sap is between 4.6 and 7 with extremes of 0.9 to 8.0. The vacuoles of plant cells usually accumulate acid and neutral salts of oxalic, citric and malic acids, and hence are often more acidic than the hyaline cytoplasm. Similarly differences in pH have been reported between cytoplasm, nuclei and granules (42) which again suggest that cells are not homogeneous with respect to hydrogen ion activity. The possibility that major differences in pH might exist between microenvironments within cells and in various tissues is suggested by a compilation of pH optima for a number of enzymes (Table VI). These values were selected to illustrate maximum rather than average pH ranges over which enzymes operate effectively. Thus the pH optimum for manganese-activated arginase is 10.2 while the

TABLE VI

pH OPTIMA OF SOME ENZYMES FROM PLANT AND ANIMAL SOURCES

Enzyme	Source	Substrate	pH Optimum	Reference
Arginase (Mn^{++})	Pigeon liver	Arginine	10.2	(31, p. 312)
Aldehyde dehydrogenase	Beef liver	Acetaldehyde	9.3	(37)
Aldehyde dehydrogenase	Yeast	Acetaldehyde	8.75	(6)
L-Hydroxy acid oxidase	Rat kidney	Pyruvate	8.0	(7)
Lactic dehydrogenase	Beef heart	Pyruvate	7.8	(32)
Fumarase	Pig heart	Fumarate	6.5	(2)
Ascorbic acid oxidase	Squash	Ascorbate	5.6	(35)
Malic dehydrogenase	Pigeon liver	Oxaloacetate	4.5	(44)
Pepsin	Gastric juice	Proteins	1.5–2.5	(29, p. 37)

optimum for pepsin is 1.5 to 2.5, depending upon the substrate. Of course many enzymes are associated with particulate fractions of cells and may not function in the same manner *in vitro* as they do endogenously. Nevertheless the observation that pH optima vary over so wide a range may have some significance, for nature is essentially conservative and it is difficult to conceive of an evolutionary process indiscriminate enough to select an enzyme with a pH optimum of 10 to function in an environment regulated at all times within 0.2 unit of pH 6.8. Certainly extracellular enzymes such as pepsin have pH optima close to acidities of the media in which they occur. In this case optimum hydrogen ion activity is 5 orders of magnitude away from neutrality.

The concept of acidity is not confined to solutions, for surfaces may be acid or alkaline depending on the material of which they are composed. For example Walling (45), using a method depending on the color phase of adsorbed indicators, found that solid cupric sulfate had an acidity of $<\sim -0.5$ as measured by the Hammett-Deyrup function. This represents conditions at the surface of the solid and is distinct from the acidity of aqueous solutions of copper sulfate which are hydrolyzed to a considerable extent. In biology, surfaces are represented by cell membranes, reticulate structures in the cytoplasm, and the interfaces of microsomes, mitochondria and nuclei. Albert (1) has suggested that the electrokinetic or zeta-potential should be used for measuring the pH values of biological surfaces, while Hartley and Roe (28) have derived an equation relating surface pH to solution pH which states that

$$\text{pH(surface)} = \text{pH(solution)} + \zeta/0.06$$

where ζ is the zeta-potential in volts. Danielli (24, 25, 26) has called attention to the "protein error" incurred in measuring the acidity of surfaces with indicators and states that this can amount to 2 pH units when the dye and protein possess opposite charges. Another example of the effect of surfaces on pH is the finding by Caley and Stoffer (16) that pH varies between 5.7 and 10.2 on mixing neutral solutions of sodium sulfate and barium chloride. The final reaction products are also neutral, but during the precipitation of the barium sulfate the hydrogen ion activity of the suspensions varies by more than 4 orders of magnitude, presumably because of the adsorption of ions by the precipitate. Thus pH changes can occur at surfaces and in solutions during the course of chemical reactions. While similar effects have not been observed in biological systems, it seems inconsistent to believe that pH would be uniform throughout the metabolizing protoplast considering the many surfaces it contains and the multiplicity of reactions that are taking place.

Thus if pH and pK can vary by several orders of magnitude, chemical reactivities with toxicants of the general type discussed here might also differ within cells and between cells of different tissues, thus leading to a degree of biological specificity. For example trichloroacetate, in contrast to iodoacetate, does not react with amino acids or plant breis under "physiological" conditions (30) although it is known to react with pyridine-alkali reagent at higher temperatures (12). This suggests the possibility that there may be specific locations within metabolizing cells that are particularly vulnerable to reagents of this type because of low pK or localized regions of high pH, so that more or less specific combinations can take place that result in their biological activity.

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THE SULFUR METABOLISM OF YEAST. I. A STUDY OF RELATIVE GROWTH OF FIVE YEASTS ON A SULFUR-FREE MEDIUM SUPPLEMENTED WITH SMALL QUANTITITES OF SULFUR COMPOUNDS[1]

David Margolis and Richard J. Block

SUMMARY

The growth responses of 5 yeasts to 39 sulfur-containing compounds supplied individually in an aerated, sulfur-free, liquid medium were studied.

The yeasts utilized inorganic sulfur (sulfate, sulfite and thiosulfate) as well as L-homocysteine, L-cystathionine, L-methionine, *S*-methyl-L-cysteine, *N*-acetylmethionine, and taurine equally well. The following compounds did not support growth in any of the yeasts examined: cysteamine, cystamine, thiolhistidine, ergothioneine, thiolmalic acid, D-ethionine, thiourea, thiamine, thiamine disulfide, and thiamine disulfide-phosphoric acid ester. The five yeasts showed individual variations in growth response to the following compounds: L-cysteine, DL-homocysteine thiolactone, L-cysteine ethyl ester, glutathione (reduced and oxidized), L-cystine, *meso*-cystine, L- and D-homocystine, DL-lanthionine, L+*meso*-lanthionine, L-djenkolic acid, D-methionine, L-ethionine, methyl methionine sulfonium chloride, L-cysteinesulfinic acid, L-cysteic acid, methionine sulfoxide and methionine sulfone.

The significance of these results in relation to the knowledge of sulfur metabolism is discussed.

Schultz and McManus (5) have investigated the availability of inorganic and organic sulfur in some 80 yeast cultures. The experiments reported here employed 5 of the yeasts tested by these investigators but included studies on some 30 additional compounds.

EXPERIMENTAL

MATERIALS AND METHODS

The five yeasts employed were: *Candida utilis* (Henneberg) Lodder [*Torula utilis* (Henneberg) Lodder], *Candida pseudotropicalis* (Cast.) Basgal (*Torula cremoris* Hammer et Cordes), *Saccharomyces cerevisiae* Hansen strain *anamensis*, *Saccharomyces cerevisiae* Hansen Rasse M, and *Zygosaccharomyces mengelicus*.[2]

Stock cultures of yeasts were maintained on 3.4 per cent proteose tryptone agar (Difco). Transfers were made to a fresh agar slant one or two days before required for an experiment to insure a supply of viable

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[2] The authority for the nomenclature of the first 3 yeasts is Lodder and Kreger-Van Rij (2, pp. 546, 512, 122) and the Fleischmann Laboratories is the authority for the latter two.

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young cells. The cells were transferred to 100 ml. of sterile 0.9 per cent NaCl solution. Only one ml. of this suspension was added to each growth tube to avoid the transfer of extraneous sulfur.

The basal medium was similar to that of Schultz and McManus (5) with the following exceptions: sucrose was substituted for glucose; 1 ml. of a trace element solution was added to 150 ml. of nutrient solution (57 mg. H_3BO_3, 36 mg. $MnCl_2 \cdot 4H_2O$, 147 mg. $ZnCl_2 \cdot H_2O$, 27 mg. $CuCl_2 \cdot 2H_2O$, 25 mg. $Na_2MoO_4 \cdot 2H_2O$ in 1 liter of water), the volume of the basal medium per tube was increased to 8 ml., the total volume of solution was 25 ml. and the level of sulfur was raised to 20 μg.

The sulfur compounds were not sterilized by autoclaving in order to avoid alteration of the molecule. Destruction during autoclaving was demonstrated as follows: sealed tubes containing nutrient solution and 20 micrograms of H_2S were autoclaved for 15 minutes at 20 pounds pressure and the solution was analyzed for its H_2S content. The analyses indicated that all of the H_2S had been oxidized. As a consequence, the solutions of sulfur compounds were kept frozen until used and the more labile compounds (viz. cysteine and reduced glutathione) were prepared fresh for each experiment.

The yeasts were grown in pyrex aeration tubes for 40 hours at 20° to 25° C. The tubes were connected in series to an aeration train and air was passed through to provide sufficient oxygen and agitation. To remove sulfur contaminants, the air was washed with freshly prepared solutions of 20 per cent zinc acetate and sodium hydroxide in series.

The yeasts were also grown in large sealed flasks on a rotary shaker at 25° C. for 40 hours to determine whether the removal of CO_2 from the air had any effect. All five yeasts gave results which were the same as those obtained by the aeration procedure.

The relative amount of growth was measured in a Klett-Summerson photoelectric colorimeter at 420 mμ after dilution of the suspension to 50 ml.

It was ascertained that the addition of 20 μg. of sulfur as sulfate per 25 ml. of nutrient solution resulted in good but not maximal growth under the above conditions. Therefore, all experiments described in this paper are comparisons of the optical density of the yeast cells produced by 20 μg. of sulfur compared with that induced by an equal quantity of Na_2SO_4-sulfur. In each experiment, a sulfate standard in duplicate was run and the optical density induced by the other compounds was compared to the sulfate standards.

Table I shows the average growth and the standard deviation obtained in 3 to 6 replicated experiments on 9 different sulfur-containing compounds. The coefficients of variation in these experiments range from 1 to 9 per cent.

TABLE I

REPRODUCIBILITY OF THE GROWTH OF CANDIDA UTILIS BASED UPON Na_2SO_4-SULFUR AS 100 PER CENT

20 μG. of sulfur as:	No. of experiments	Mean	Standard deviation
$SO_3^=$	3	109	3
$S_2O_3^=$	6	98	9
L-Cystine	5	89	8
L-Methionine	3	108	7
L-Cysteine	5	93	6
Glutathione (reduced)	3	101	1
L-Cysteic acid	4	104	6
Taurine	4	104	4
L-Cysteinesulfinic acid	5	86	6

RESULTS

The experimental findings are presented in Table II. Attention is called particularly to those compounds to which the yeasts showed individual variations.

C. utilis appears to be the most versatile. In the presence of the L-isomer, it is able to metabolize approximately 50 per cent of the sulfur of D-homocysteine and of D-ethionine and all of the S of D-cystathionine, D-allocystathionine and D-methionine. D-Homocystine, at least in the presence of L-homocystine, produces cells of unusually large size and density which accounts for the apparent value of 143 per cent.

C. pseudotropicalis differs from *C. utilis* in its inability to metabolize the disulfides and D-amino acids containing a thioether linkage.

S. cerevisiae anamensis is unable to metabolize L-cysteine, L-cysteinesulfinic acid and L-cysteic acid but is able to assimilate both reduced and oxidized glutathione. Like *C. pseudotropicalis*, it is unable to assimilate D-isomers or disulfides except for oxidized glutathione.

S. cerevisiae Rasse M is unable to metabolize either form of glutathione.

Z. mengelicus resembles *S. cerevisiae* Rasse M in its ability to metabolize sulfur compounds except for the poor growth by the former on DL-allocystathionine and L-cysteic acid.

DISCUSSION

In order to facilitate an understanding of the various interrelationships of the compounds discussed in this paper, Figure 1 is presented to show the known and assumed metabolic pathways of sulfur (1, 3, 4, 6). Figure 2 is a condensation of the pathways shown in Figure 1 and indicates the individual differences found in the five yeasts studied.

Growth by a yeast with a particular source of organic sulfur does not preclude its prior conversion to inorganic sulfur. The inability to grow may be due to toxicity of the compound in question, failure to penetrate

TABLE II

COMPARATIVE OPTICAL DENSITY OF YEAST CULTURES AFTER 40 HOURS' GROWTH WITH VARIOUS SOURCES OF SULFUR; EXPRESSED AS PER CENT OF GROWTH ON AN EQUIMOLAR QUANTITY OF SULFATE SULFUR

Source of S	*Candida utilis*	*Candida pseudotropicalis*	*S. cerevisiae*		*Zygosaccharomyces mengelicus*
			anamensis	Rasse M	
Inorganic sulfur					
Sulfate	100	100	100	100	100
Sulfite	109	105	109	99	113
Thiosulfate	98	103	107	98	104
Organic sulfur					
Thiols					
L-Cysteine	93	91	30	109	82
DL-Homocysteine	71	67	41	49	46
DL-Homocysteine thiolactone · HCl	23	65	23	40	37
L-Cysteine ethyl ester · HCl	43	29	18	48	19
Cysteamine · HCl	19	27	19	2	6
Thiolhistidine	0	16	6	3	0
Ergothioneine · HCl	8	25	10	21	0
Thiomalic acid	15	14	8	25	0
Thiourea	0	8	5	9	0
Glutathione (reduced)	101	111	80	15	34
Disulfides					
Glutathione (oxidized)	114	99	88	6	26
L-Cystine	89	3	21	0	20
meso-Cystine	88	11	8	0	8
DL-Homocystine	143	5	9	11	7
L-Homocystine	100	0	9	15	18
Cystamine	14	0	13	10	12
Thioethers					
DL-Cystathionine	97	49	45	87	70
DL+Allocystathionine*	88	43	63	94	32
DL-Lanthionine	67	13	9	6	12
L+*meso*-Lanthionine*	59	16	11	10	14
L-Methionine	108	103	94	89	98
D-Methionine	95	73	12	14	13
DL-Ethionine	37	11	5	29	2
L-Ethionine	48	24	0	43	8
S-Methyl-L-cysteine	96	121	106	118	93
N-Acetylmethionine	69	60	61	65	63
Oxidized sulfur compounds					
L-Cysteinesulfinic acid	86	74	24	82	89
L-Cysteic acid	104	92	18	64	30
Taurine	104	85	75	84	71
Methionine sulfoxide	103	108	65	69	57
Methionine sulfone	101	89	19	42	42
Miscellaneous					
Methyl methionine sulfonium chloride	40	46	79	47	74
L-Djenkolic acid	55	0	6	23	12
Thiamine · HCl	4	7	2	15	0
Thiamine disulfide**	18	26	3	0	12
Thiamine disulfide-phosphoric acid ester**	0	16	6	17	15

* Obtained from Mann Research Laboratories, New York, N. Y.

** Kindly supplied by E. Merck AG., Darmstadt, Germany.

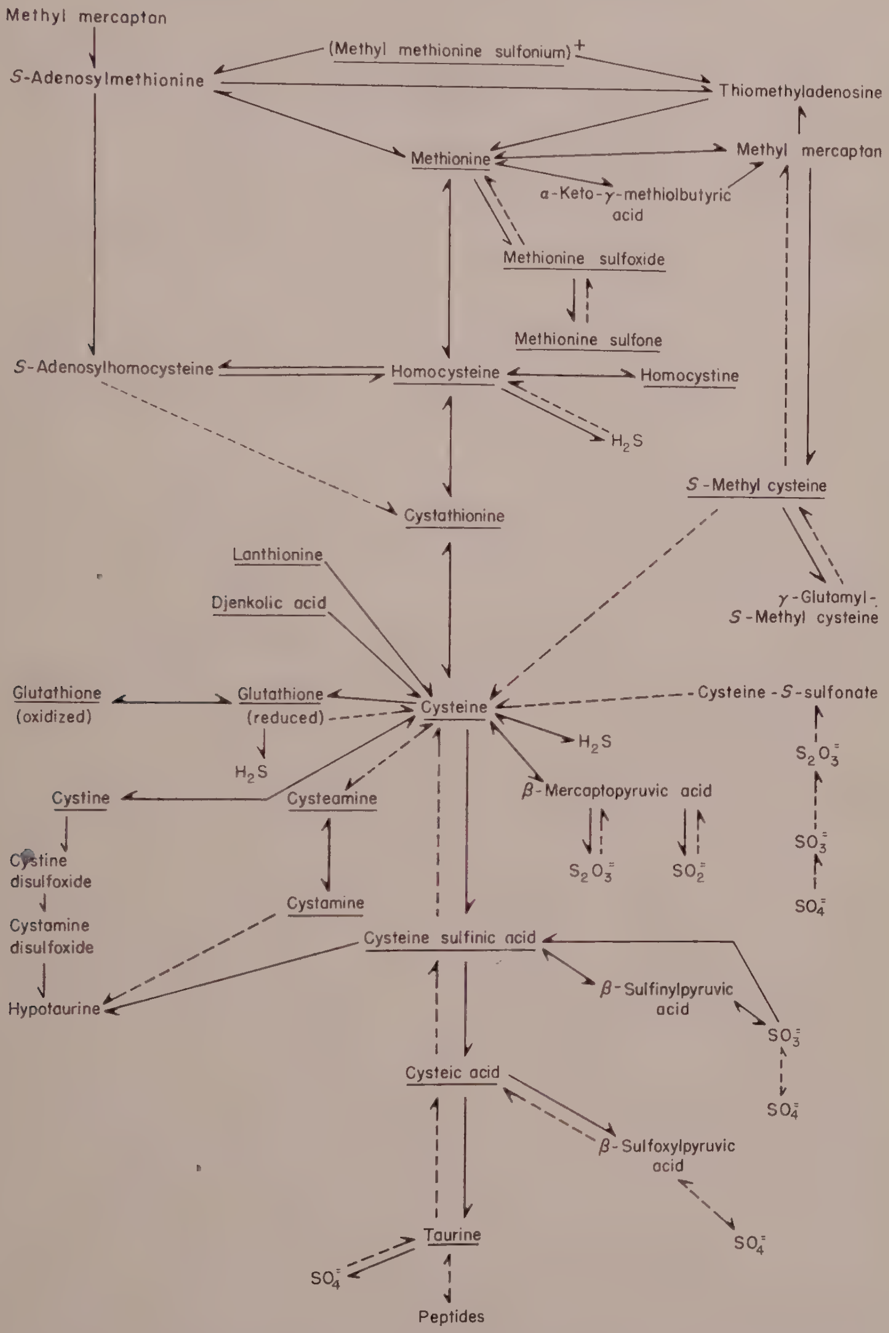

FIGURE 1. The metabolic pathways of sulfur. The sulfur-containing compounds tested in this experiment are underlined; dotted lines indicate doubtful reactions.

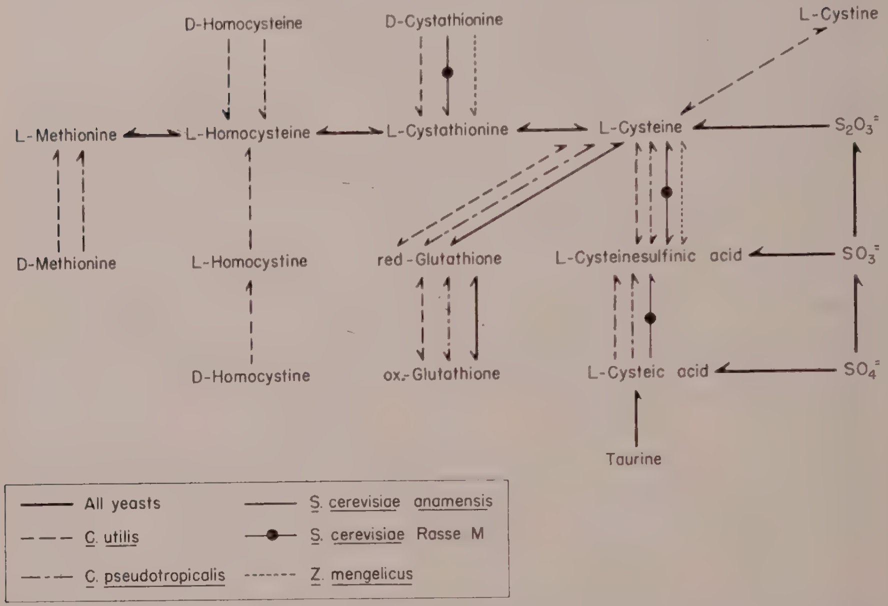

FIGURE 2. Possible metabolic pathways of sulfur-containing compounds in yeasts. Arrows indicate those yeasts which appear to be able to carry out the transformation indicated. The other yeasts are unable to do so under the experimental conditions.

the cell wall, inability to synthesize the enzymes appropriate for its metabolism, and/or the possible formation of volatile sulfur-containing products.

Attention is called particularly to the behavior of *S. cerevisiae anamensis* which appears to be unable to assimilate cysteine, cysteinesulfinic acid and cysteic acid. This finding casts doubt upon the mechanism of incorporation of inorganic sulfur into an organic metabolite. As yet, there is no substantial demonstration of this mechanism but it is postulated (Fig. 1) that thiosulfate reacts with a three-carbon acceptor to form cysteine via cysteic acid or cysteine-*S*-sulfonic acid. Oxidation of cysteine to cystine does not explain the poor growth on the former because both homocysteine and reduced glutathione support good growth. The fact that *S. cerevisiae anamensis* as well as the other yeasts are able to utilize *S*-methyl-L-cysteine suggests that this compound may be an important intermediate in the conversion of inorganic S to organic S.

S. cerevisiae Rasse M and *Z. mengelicus* typify the rather specific differences in metabolism of various yeasts. These organisms, in contrast with the closely related *S. cerevisiae anamensis*, are unable to utilize glutathione but can assimilate cysteine. When the former yeasts were fed $Na_2S^{35}O_4$ and the extracts were examined by paper chromatography, S^{35}-cysteine and

S^{35}-glutathione were found. Glutathione, therefore, apparently does not penetrate the cell walls of *S. cerevisiae* Rasse M and *Z. mengelicus*.

In contrast to our findings, Schultz and McManus (5) reported that both cysteine and sulfide were utilized by the yeasts employed in this study. These discrepancies may be explained by the fact that Schultz and McManus added the sulfur-containing compounds to the medium before autoclaving, which procedure converted some H_2S to sulfate and probably caused considerable oxidation of cysteine.

ACKNOWLEDGMENT

We are indebted to Dr. Lawrence Atkin, Assistant Director of Research, the Fleischmann Laboratories, Stamford, Conn., for the yeast cultures.

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AMINO ACID COMPOSITION OF THE SERUM PROTEINS. I. THE FRACTIONATION OF BOVINE SERUM PROTEINS BY AMMONIUM SULFATE AND THE COMPARATIVE AMINO ACID COMPOSITION OF THE FRACTIONS[1,2]

STEPHEN KELLER AND RICHARD J. BLOCK

SUMMARY

The proteins of bovine serum were separated by fractional precipitation with ammonium sulfate. The preparations so obtained were then analyzed for nitrogen, amino acids and for protein components by paper electrophoresis. The analyses indicated that several groups of amino acids (cystine and alanine; aspartic acid and lysine; tyrosine, serine and glycine) have molar ratios that are relatively constant in all the fractions, even though the absolute values of the amino acids present in the various preparations vary considerably. These findings are interpreted as being compatible with the hypothesis of amino acid "anlagen," that is, of some structures of relatively constant composition in all the serum proteins.

INTRODUCTION

Although the serum proteins have been studied for over a century' insight into their interrelations is lacking. The many hypotheses that have been proposed to explain the structure and behavior of these proteins range on the one hand from the dissociable complex hypothesis of Sørenson (11) and the "anlage" hypothesis of Block (1, 2) to those which hold that these proteins are discrete entities which have no relation whatsoever to one another. This latter view was first proposed by Lewith (8). He believed that there were only an albumin and a globulin in mammalian blood sera. This view was gradually modified until Tiselius (12) definitely showed the complexity of the globulin fraction. Even today, most workers still regard human blood serum as consisting of only albumin, alpha$_1$-, alpha$_2$-, beta- and gamma-globulins. Therefore, the accepted view today is that the serum proteins are comprised of these five major fractions, existing as well-defined, completely characterized entities along with a few minor components.

The experiments described in this paper were undertaken to throw further light on the interrelationships, if any, among the serum protein fractions obtained by salting out with ammonium sulfate.

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[1] Aided by grant H-2487 (c), National Heart Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

[2] Part of a thesis submitted by Stephen Keller in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biochemistry, Rutgers University, New Brunswick, New Jersey.

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EXPERIMENTAL

The proteins of beef serum were isolated by means of fractional ammonium sulfate precipitation. This method, originated by Méhu (9), was improved by Cohn *et al.* (5). The latter investigators employed dialysis of serum against salt solutions of increasing ionic strength, and characterized each of the fractions by electrophoretic procedures.

In the present investigation, beef serum proteins from 600 ml. of defibrinated blood were subjected to fractional ammonium sulfate precipitation in steps of 7 or 8 per cent of saturation (referred to 0° C.). The serum was dialyzed against these ammonium sulfate solutions, and protein precipitates were obtained in the range from 20 to 72 per cent of saturation. The dialysis in each instance was allowed to continue for four days at 5° C. The precipitates so obtained were filtered and washed with ammonium sulfate solutions of the same concentration as employed in the precipitation. The precipitates were dissolved in small volumes of water, and were dialyzed against distilled water for at least four days in the cold to remove the salt. Both barium chloride and Nessler's reagent were employed to ascertain freedom from sulfate and ammonium ions, respectively. The resulting salt-free protein solutions were lyophilized.

TABLE I

Nitrogen Analysis and Classes of Proteins Separated by Electrophoretic Mobilities in Fractions Precipitated from Bovine Serum by Different Concentrations of Ammonium Sulfate

Fraction	Ammonium sulfate as per cent of saturation	Per cent nitrogen*	Protein components in per cent of total			
			γ-globulins	β-globulins	α-globulins	Albumins
A	28	12.5	80.4	19.6	0	0
B	35	13.4	72.2	27.8	0	0
C	42	12.4	52.2	24.3	23.5	0
D	50	10.5	20.7	37.7	23.2	18.4
E	57	12.2	0	25.1	38.8	36.1
F	65	12.1	0	22.0	27.2	50.8
G	72	8.7	0	0	0	100.0

* Uncorrected for moisture and ash.

Each of the lyophilized preparations was subjected to paper electrophoresis to ascertain the types of the serum proteins present (4, pp. 523–528, 554–579). The protein components of each fraction are given in Table I.

Nitrogen was determined by the Kjeldahl procedure using the Kirk (7) steam distillation method. The amino acids were analyzed by paper chromatography (3, pp. 71–87).

The amino acid values as millimoles per 1.6 g. of nitrogen are given in

TABLE II

APPROXIMATE AMINO ACID COMPOSITION OF BOVINE SERUM PROTEINS OBTAINED BY SALTING OUT WITH AMMONIUM SULFATE
(MILLIMOLES OF AMINO ACID PER 1.6 G. OF N)

	Protein fraction						
	A	B	C	D	E	F	G
Cystine	2.7 2.9	2.9 2.9	2.3 2.7	2.7 2.6	3.0 3.2	4.9 5.8	2.6 3.3
Alanine	4.9 5.4	5.4 4.4	— —	4.3 3.7	4.5 5.3	6.5 8.3	5.5 6.1
Lysine	5.6 5.1	5.0 4.9	4.9 5.7	4.0 4.0	5.3 5.5	5.2 7.0	6.2 6.0
Aspartic acid	6.9 8.1	7.4 5.3	5.7 5.8	4.3 5.3	5.8 6.0	9.5 8.4	8.1 6.4
Phenylalanine	2.6 2.1	2.9 2.4	3.8 3.2	2.7 3.1	3.4 2.9	3.2 5.5	3.1 2.7
Tyrosine	3.3 3.1	2.7 2.9	— —	1.2 1.7	2.9 2.2	1.6 3.3	2.0 2.6
Serine	8.8 6.8	6.6 4.9	8.5 7.3	3.4 3.4	2.6 3.9	2.9 4.6	2.8 2.6
Glycine	8.2 9.0	7.1 5.6	7.0 6.2	5.3 4.3	3.9 4.2	4.8 4.8	3.7 3.1
Histidine	1.4 1.5	1.4 1.7	2.1 1.7	— —	1.6 1.6	2.8 3.2	— —
Methionine	0.72 0.67	1.6 1.6	0.85 0.79	0.76 0.76	0.76 0.76	0.71 0.71	— —
Threonine	4.4 5.0	5.4 4.1	4.9 4.9	4.9 5.0	4.7 3.9	6.4 6.0	4.8 5.0
Glutamic acid	19.0 15.4	16.2 12.9	17.0 12.9	11.6 11.7	12.0 9.7	18.2 15.2	17.3 14.9

Table II. It should be noted that all determinations for any group of amino acids were carried out simultaneously in order to increase the comparative value of the results.

RESULTS

The nitrogen contents of the various fractions varied from 8.7 per cent to 13.4 per cent (Table I). The first three fractions had the highest nitrogen contents, and their electrophoretic patterns showed them to be rich in gamma globulins. The N content of the proteins in fraction G was lower than the gamma globulin-rich fractions. The next two fractions showed an increasing amount of albumin, and a concomitant increase in nitrogen.

The last fraction was the lowest in nitrogen, and its electrophoretic pattern showed it to be homogeneous albumin. The indication is, therefore, that this fraction is the glycoprotein component of the serum albumin. Such a protein has been isolated from serum albumin by Hewitt (6) by a different procedure.

From the duplicate amino acid determinations of the protein fractions (Table II) the molar ratios between corresponding values of two amino acids were calculated. An analysis of variance was carried out on the resulting ratios to test their constancy within each group. In each case, the variance ratio was calculated, and compared with the theoretical ratio, or F value for a 5 per cent level (10). The molar ratios within each of three groups of amino acids (phenylalanine-lysine-aspartic acid, cystine-alanine, and tyrosine-serine-glycine) were found to be statistically constant for the seven protein fractions. Those in the methionine-threonine-glutamic acid group were statistically different.

There are at least three groups of amino acids which appear to have relatively constant molar ratios in all of the different fractions isolated, even though the absolute values varied considerably. Thus, the average molar ratio of the lysine to aspartic acid is 1.0:1.3 (Table III).

A second group consists of cystine and alanine, the distribution of which in the serum proteins gave an average molar ratio of 1.0:1.7 and a range of from 1.4 to 2.0. A third group of amino acids, the distribution of which is fairly constant consists of tyrosine, serine, and glycine with an average molar ratio of 1.0:1.9:2.3. There is a general trend for these latter amino acids to decrease in amount from the first to the last protein fraction. It is such a systematic variation that prompted the early workers to promulgate their various hypotheses regarding proteins as being complexes. These results are to be contrasted with the remaining amino acids for which

TABLE III

MOLAR RATIOS OF THE GROUPS OF RELATED AMINO ACIDS

Amino acids	Protein fraction							Average molar ratio
	A	B	C	D	E	F	G	
Aspartic acid/lysine	1.4	1.3	1.1	1.2	1.1	1.5	1.2	1.3
Phenylalanine/lysine	0.44	0.54	0.67	0.73	0.59	0.71	0.48	0.59
Alanine/cystine	1.8	1.7	—	1.5	1.6	1.4	2.0	1.7
Serine/tyrosine	2.5	2.1	—	2.5	1.4	1.6	1.2	1.9
Glycine/tyrosine	2.7	2.3	—	3.5	1.7	2.2	1.5	2.3
Molar ratios of the unrelated amino acids								
Threonine/methionine	6.8	3.1	6.0	6.8	5.7	8.7	—	6.2
Glutamic acid/methionine	24.8	10.5	18.2	15.3	14.4	23.5	—	17.8

analyses were made, namely, glutamic acid, threonine, histidine and methionine. These amino acids do not appear to follow any particular type of distribution. It may be that one or more of the independent amino acids also exist as "anlagen" with amino acids for which analyses were not made.

DISCUSSION

The observation that at least three groups of amino acids have constant molar ratios in all of the fractions indicates the presence of some units of essentially the same amino acid composition in all the serum proteins. Although each such unit, or "anlage," appears to be present in different absolute amounts, its presence may indicate that all serum proteins are chemically related in spite of their different sites of formation and the changes in their relative quantities in health and disease. As a first approximation, each serum protein may be considered to be related to every other serum protein by certain amino acids which exist in relatively constant ratio and differentiated from each other by variations in the absolute quantities of the "anlage" amino acids and by those amino acids which do not appear to be present in "anlagen."

It is possible that such common peptide units represent a biological phenomenon in many protein systems. This is understandable from an evolutionary and developmental point of view and may be a manifestation of common template units having their roots in ancestral proteins.

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We wish to thank Prof. E. V. McCollum for calling our attention to the investigations of Méhu.

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AMINO ACID COMPOSITION OF THE SERUM PROTEINS. II. THE FRACTIONATION OF HUMAN SERUM PROTEINS BY CELLULOSE ION-EXCHANGE CHROMATOGRAPHY AND THE COMPARATIVE AMINO ACID COMPOSITION OF THE FRACTIONS[1,2]

Stephen Keller and Richard J. Block

SUMMARY

The proteins of human serum were separated by diethylaminoethylcellulose ion-exchange chromatography. This procedure yielded 15 protein components, nine of which were homogeneous and the rest were heterogeneous by paper electrophoresis.

Amino acid analyses of nine of the fractions revealed the presence of five groups of amino acids which had a relatively constant molar ratio even though the fractions were often electrophoretically heterogeneous and were obtained from the column under widely different conditions. Two of these groups possessed a pair of amino acids in a molar ratio of approximately 1:2.2, while the amino acids in the three remaining groups had ratios of 1:1. These constant molar ratios suggest the existence of units of similar structure in all of the serum proteins and appear to support the idea that the serum proteins, even though they are synthesized in a number of different organs, may have all developed from peptides of relatively constant amino acid composition, that is from amino acid "anlagen."

INTRODUCTION

It has been previously shown (7) that when bovine serum proteins are fractionated by increasing increments of ammonium sulfate the resulting protein preparations contain a number of amino acids which are present in approximately constant molar ratios while other amino acids vary considerably. This is true irrespective of the protein fraction's solubility in aqueous ammonium sulfate or its composition as indicated by electrophoresis. These results are interpreted as suggesting the presence of amino acid "anlagen" or of some structures (peptides) of constant composition throughout the serum proteins.

In view of the electrophoretic heterogeneity of the protein fractions obtained by ammonium sulfate fractionation (7), it was considered advisable to pursue the problem employing another method of protein fractionation and another mammalian serum. Therefore, human serum

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[1] Aided by grant H-2487 (c), National Heart Institute, National Institutes of Health, U. S. Public Health Service, Bethesda, Maryland.

[2] Part of a thesis submitted by Stephen Keller in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Physiology and Biochemistry, Rutgers University, New Brunswick, New Jersey.

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was fractionated by the elegant method of Peterson and Sober (10) which employs chromatography on a column of modified cellulose.

EXPERIMENTAL

Cellulose ion-exchange chromatography. The proteins of human serum were fractionated by column chromatography employing the anion-exchanger diethylaminoethyl cellulose prepared from Solka-Floc BW-200 according to the method of Peterson and Sober (10). Fifteen grams of DEAE-SF cellulose, suspended in a 0.005M phosphate buffer at pH 7.0, were placed in a 2.2 × 25-cm. column. Fifty ml. of pooled human serum, were dialyzed for 24 hours against one liter of 0.005M phosphate buffer. The dialyzed serum was applied to the column which had been previously equilibrated with the starting buffer (0.005M phosphate). The proteins were fractionated by a series of buffers, similar to those used by Sober *et al.* (16), involving a gradual increase in ionic strength and a decrease in pH.

Gradient	*Time (hours)*	*Tube No.*	*Buffer composition*
I	0	0	0.005M Phosphate buffer, pH 7.0
II	70	140	0.02M Phosphate buffer, pH 6.0
III	123	246	0.05M NaH_2PO_4, pH 4.5
IV	170	340	0.05M NaH_2PO_4 + 0.02M NaCl, pH 4.5
V	240	480	0.05M NaH_2PO_4 + 0.05M NaCl, pH 4.5
VI	310	620	0.05M NaH_2PO_4 + 0.10M NaCl, pH 4.5

The mixing flask volume was 210 ml. and the flow rate was adjusted to 0.25 to 0.30 ml. per minute by varying the hydrostatic head. The volume of each fraction collected was 5 ml. Every third tube collected was read in a Beckman DU spectrophotometer in the ultraviolet at 280 mμ using a 1-cm. quartz cell. Eluates corresponding to each of the protein peaks were dialyzed free of salts, lyophilized and the components identified by paper electrophoresis (4, p. 523–532, 554–579).

Amino acid analyses. Aspartic acid, glutamic acid, serine, glycine and threonine were determined by the method described previously (7). The amino acids in group B (cystine, lysine, histidine, arginine, alanine, tyrosine, methionine, valine, phenylalanine, leucine plus isoleucine) were estimated as follows: one-μl. aliquots of the hydrolyzates and standard solutions were applied to sheets of Whatman No. 3 filter paper, 22 × 18 inches. The amino acids were separated by descending chromatography using a mixture of *n*-butanol-glacial acetic acid-water = 450:50:125 v/v as the developing solvent. The quantity of each amino acid was then estimated by the maximum density method (3, p. 71–87).

RESULTS

The separation of the human serum proteins by DEAE-SF-cellulose chromatography yielded 15 protein peaks (Fig. 1). The first six peaks

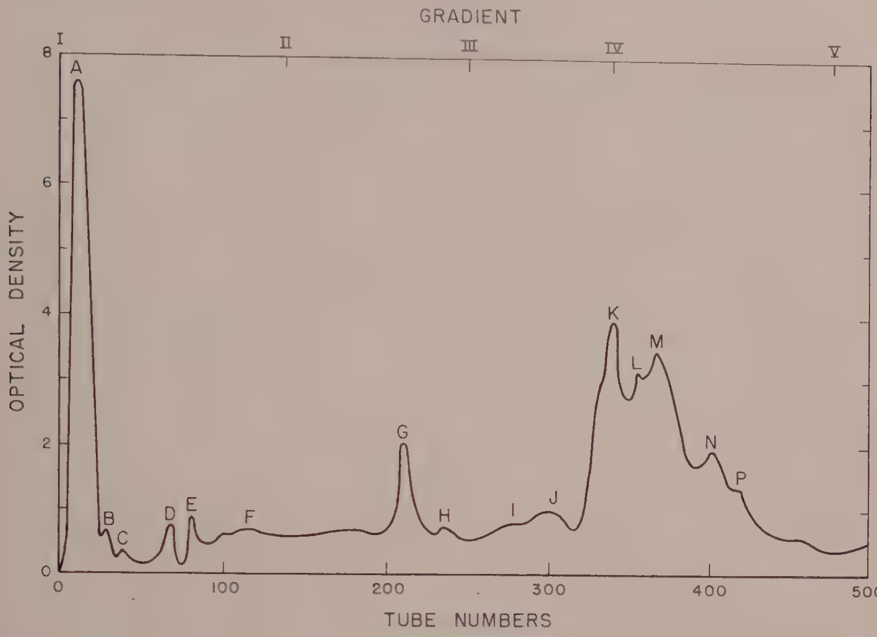

FIGURE 1. Separation of human serum proteins on DEAE-SF-cellulose column.

eluted from the column were composed of gamma-globulins with at least two different electrophoretic mobilities. Fractions G and H contained β_1-globulins, and fraction I was composed of β_2-globulins. Fraction J was a mixture of β_2- and α_2-globulins. Fractions K and L were chiefly albumin, while M and N were rich in albumin but with increasing amounts of α_1- and α_2-globulins. Fraction P was richest in α_2-globulins (Table I). A comparison of the nitrogen distribution and electrophoretic patterns can be seen in Table I. γ_1-, β- and α_2-globulins contain relatively small quantities of nitrogen; in fact, the lowest nitrogen of any protein fraction in the series is fraction H, a β_1-globulin. These globulins are known to be conjugated with lipids, metals and carbohydrates. Fractions K, L and M have the highest nitrogen contents, and correspond to the fractions richest in albumin.

Amino acid analyses of nine fractions were performed, and the results are given in Table II. These data indicate the presence of at least five groups of amino acids, each characterized by having its component amino acids in fairly constant molar ratios (Table III and Fig. 2). One pair consists of arginine and lysine with an average molar ratio of 1.0:2.3. A second pair is leucine plus isoleucine and aspartic acid in the ratio 1.0:1.1. Lysine and arginine almost fit into this group. The third pair consists of cystine and alanine with an average molar ratio of 1.0:2.2. This group also

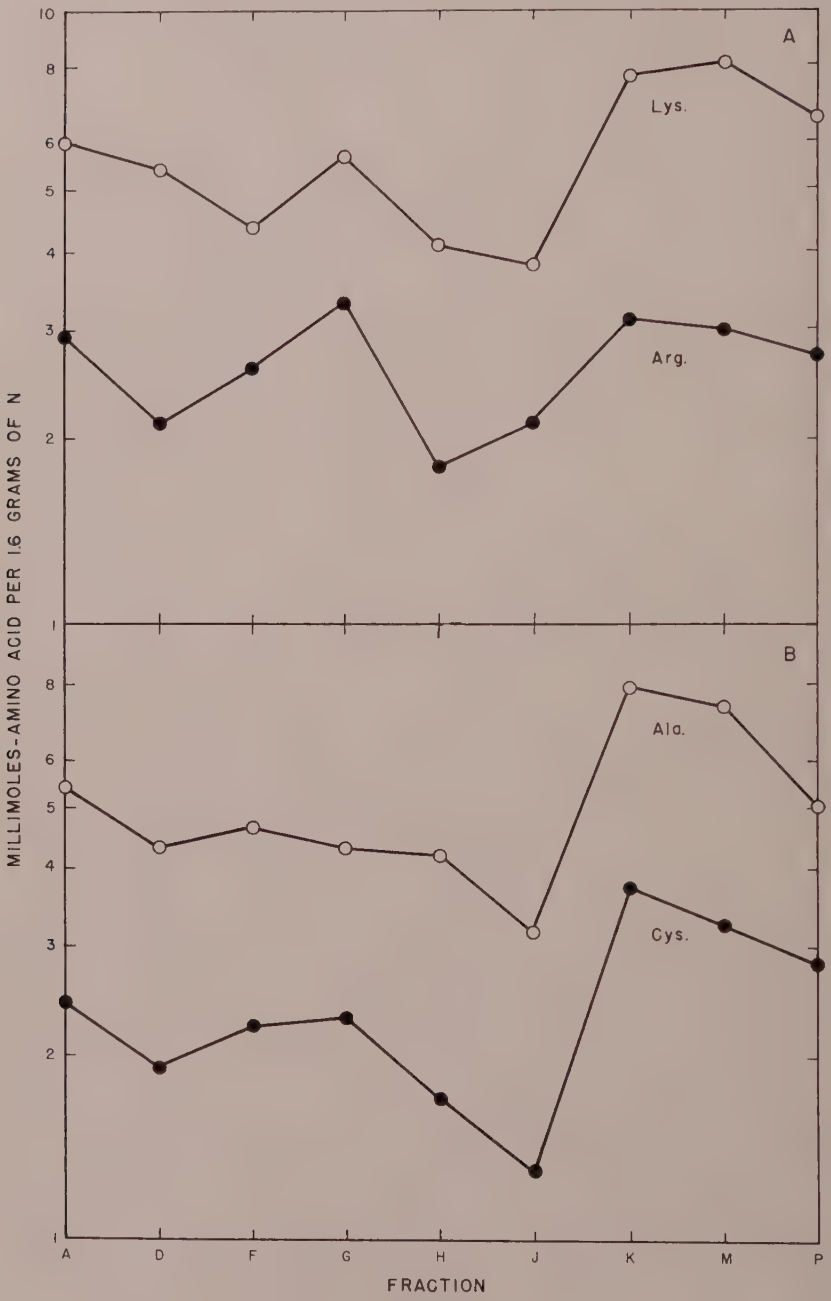

FIGURE 2. Interdependent amino acids.

TABLE I

Electrophoretic Determination of Composition of the Human Serum Protein Fractions

Fraction	Per cent nitrogen*	Components (% of each fraction)						
		γ_2	γ_1	β_2	β_1	α_2	α_1	Albumins
A	13.5	100						
B	—		100					
C	9.4		100					
D	9.0		100					
E	—		100					
F	5.9		100					
G	6.7				100			
H	5.4				100			
I	8.4			100				
J	9.8			66.4		33.6		
K	15.2			10.1				89.9
L	15.0					11.2	14.7	74.1
M	15.0			4.8		13.6	13.3	68.3
N	13.8			12.1		27.1	16.7	44.1
P	12.0					57.3		42.7

* Uncorrected for moisture and ash.

closely resembles the first. A fourth group of interrelated amino acids consists of serine and glycine, with an average molar ratio of 1.0:1.1, respectively. This pair shows a general trend to decrease in amount from the first to last fraction and thus resembles the findings on bovine serum proteins (7). Threonine and valine may also be placed into a separate group. The molar ratio of this pair of amino acids is quite constant. The remaining amino acids, tyrosine, glutamic acid, histidine, methionine and phenylalanine, seem to vary independently; three of these are shown in Figure 3. These amino acids may also exist as "anlagen" with amino acids for which analyses were not made.

The interdependent amino acids, cystine-alanine, lysine-aspartic acid, serine-glycine were found previously in bovine serum proteins fractionated by ammonium sulfate (7). Glutamic acid and histidine in both types of sera appear to be independent of any of the other amino acids for which analyses were made. In fact the only amino acid which shows a markedly different pattern in bovine and in human serum proteins is tyrosine. This amino acid conforms to the serine-glycine pattern in bovine serum proteins, but seems to be randomly distributed in human serum proteins.

An analysis of variance of the molar ratios was calculated as described previously (7). These results confirm the constancy of the various molar ratios in the above groups of amino acids. When an analysis of variance was calculated between two amino acids, each belonging to a different group (e.g. cystine and glycine), the calculations showed that there was no constancy in the molar ratios. Thus, it appears likely that amino acids

TABLE II

AMINO ACID COMPOSITION OF THE HUMAN SERUM PROTEIN FRACTIONS
(MILLIMOLES OF AMINO ACID PER 1.6 G. N)

	Protein fraction								
	A	D	F	G	H	J	K	M	P
Cystine	2.4 2.4	2.0 1.8	2.4 1.9	2.4 2.1	1.8 1.5	1.3 1.3	3.8 3.6	3.3 3.3	2.7 2.8
Alanine	5.2 5.5	4.6 3.9	5.4 3.8	4.8 3.8	4.7 3.6	2.9 3.4	8.4 7.2	7.5 7.1	4.7 5.2
Arginine	3.0 2.7	2.1 2.0	3.5 1.6	3.4 3.1	1.8 1.8	2.3 1.8	3.1 3.0	3.2 2.8	2.9 2.5
Lysine	6.2 5.8	5.3 5.5	4.6 4.1	6.4 5.0	4.2 3.9	3.9 3.6	8.4 6.9	8.5 7.5	6.7 6.5
Aspartic acid	7.1 5.6	5.7 4.8	2.7 2.7	5.3 4.1	4.8 3.9	4.1 3.8	5.9 6.2	6.3 5.3	6.4 5.7
Leucine + isoleucine	5.4 5.0	5.7 4.8	3.8 2.7	4.3 4.3	3.9 3.6	3.8 4.2	5.9 6.1	6.0 5.3	5.9 5.7
Serine	5.7 7.3	6.0 5.7	3.3 3.8	2.9 2.9	3.0 2.7	3.6 2.9	2.3 2.3	2.7 2.7	3.6 2.9
Glycine	5.2 6.5	5.9 5.7	3.3 3.8	3.8 3.8	3.8 3.3	3.6 4.1	1.7 2.2	2.6 2.1	3.5 3.2
Threonine	6.2 6.1	4.1 4.3	3.3 2.7	2.6 3.1	3.3 3.9	3.8 4.3	3.8 3.8	3.2 2.8	3.6 4.3
Valine	7.3 7.7	5.5 5.0	2.7 2.7	3.3 4.1	3.6 5.3	3.9 4.3	4.0 4.2	3.3 4.3	5.3 5.3
Tyrosine	3.3 2.4	2.4 3.2	1.0 1.4	1.7 1.9	3.0 3.3	1.6 1.5	1.9 1.3	2.0 1.7	2.9 2.4
Histidine	1.5 1.5	2.1 1.8	3.8 3.0	3.3 2.6	3.0 2.1	2.6 2.0	2.3 2.1	3.4 2.8	3.1 3.9
Glutamic acid	14.7 14.0	15.0 12.6	7.6 7.6	6.7 5.0	6.5 5.0	10.4 8.5	16.0 19.2	15.8 17.5	16.0 19.0
Phenylalanine	2.4 2.4	3.9 3.0	— —	— —	3.3 —	2.0 2.3	2.5 3.4	— —	3.6 3.3
Methionine	0.47 0.83	— —	0.43 —	0.84 —	— —	0.41 0.33	0.53 0.63	0.64 —	— —

appear in definite groups, and that the different groups of amino acids vary independently of each other.

DISCUSSION

The amino acid heterogeneity of some of the globulin fractions is shown in Table II. Porter and Press (11) and Tiselius *et al.* (17) obtained several

TABLE III

MOLAR RATIOS OF THE GROUPS OF RELATED AMINO ACIDS

Amino acids	Protein fraction									Average molar ratio
	A	D	F	G	H	J	K	M	P	
Lysine/arginine	2.1	2.7	1.9	1.8	2.3	1.9	2.6	2.7	2.5	2.3
Aspartic acid/leucine + isoleucine	1.2	1.0	0.9	1.1	1.2	1.0	1.0	1.1	1.1	1.1
Lysine/leucine + isoleucine	1.2	1.0	1.4	1.4	1.1	0.9	1.3	1.4	1.1	1.2
Alanine/cystine	2.3	2.3	2.3	1.9	2.6	2.4	2.1	2.3	1.9	2.2
Glycine/serine	0.9	1.0	1.0	1.3	1.3	1.2	0.9	0.9	1.0	1.1
Valine/threonine	1.3	1.3	0.9	1.3	1.3	1.0	1.1	1.3	1.4	1.2
	Molar ratios of the unrelated amino acids, based on tyrosine									
Histidine/tyrosine	0.6	0.7	3.1	1.7	0.8	1.5	1.5	1.7	1.3	1.4
Glutamic acid/tyrosine	5.2	4.8	6.8	3.3	1.6	6.1	11.8	8.9	6.7	6.1

γ-globulin fractions by means of column chromatography. Saifer and Corey (14) found three fractions in human γ-globulins by electrophoresis and Müller-Eberhard *et al.* (8) obtained both high and low molecular weight fractions from human γ-globulin by a combination of sedimentation and electrophoresis. Derrien *et al.* (6) reported salting-out curves for human serum which indicated the presence of several components in the albumin region as well as a multiplicity of globulins. Further evidence for the complexity of the albumin region was obtained by Tiselius *et al.* (17) who demonstrated at least three fractions in bovine serum albumin. Electrophoresis has also been used to show the compound nature of the albumin fraction. Saifer and Corey (13) found four components in bovine serum albumin using the moving boundary electrophoresis, while Raacke (12) showed the presence of at least two components by zone electrophoresis on starch.

One of the more interesting implications of the present study was the isolation of different γ- and β-globulins. Fractions A, D and F had the electrophoretic mobility of γ-globulins (Table I) but each had a different and characteristic amino acid composition (Table II). The two β-globulins obtained, fractions G and H, also had characteristic compositions. Several albumin-rich fractions were also found but, due to their electrophoretic complexity, an interpretation of the differences in their composition is not feasible at present.

The fact that there are a number of amino acids, the molar ratios of

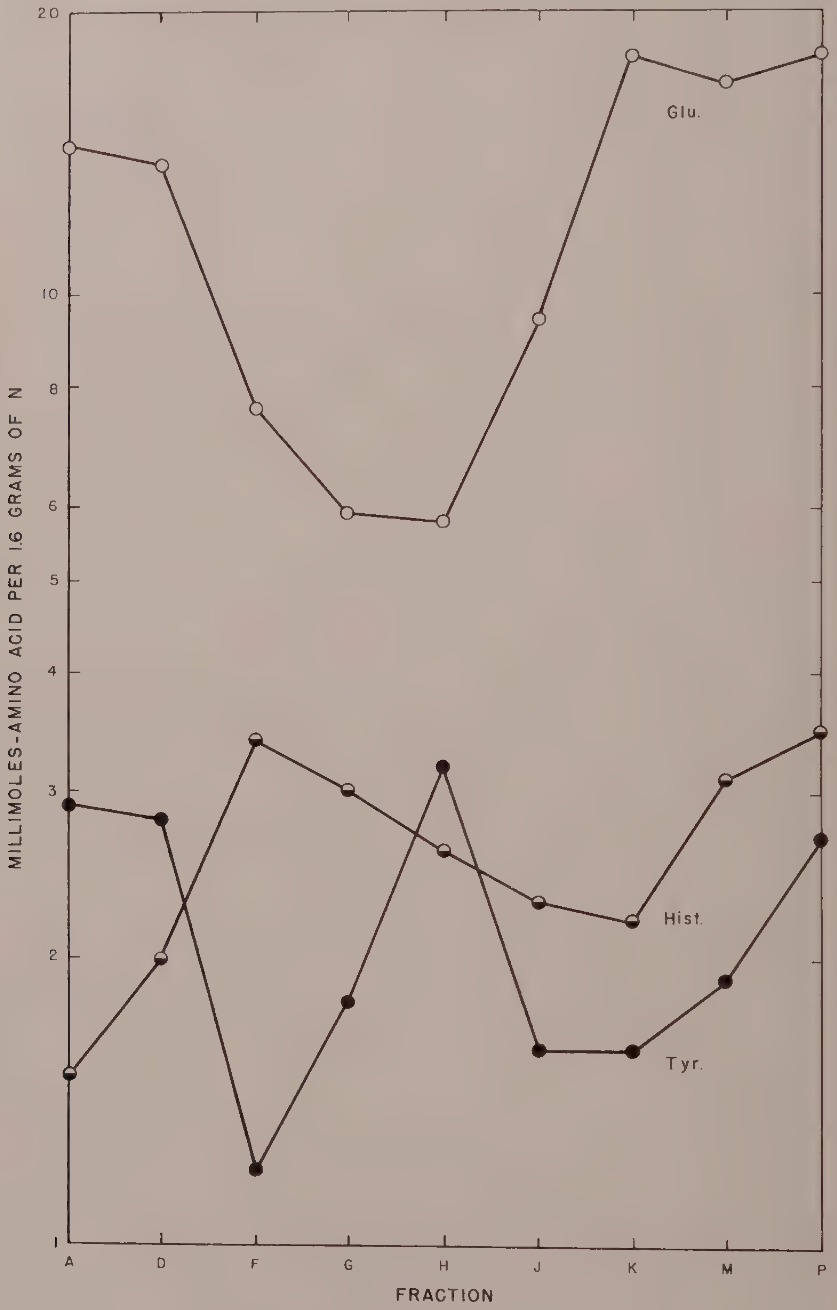

FIGURE 3. Independent amino acids.

which are essentially constant through the entire range of serum proteins, may be evidence for the presence of amino acid "anlagen" or common amino acid (peptide) units in *all* of the serum proteins. The analyses inindicate that there are at least five such groups of *interdependent* amino acids in human serum proteins (Table III). The physical, chemical and immunological properties of the serum proteins may be explained, by differences in the relative absolute quantities of those peptides which contain the interdependent amino acids, by the position of each amino acid along the peptide chain, by the spatial configuration of the proteins and by the quantities of the independently variable amino acids.

The observation that all serum protein fractions obtained by column chromatography contain certain groups of interdependent amino acids is in agreement with earlier conclusions (1, 2, 7, 15). However, Cummings and Flynn (5) and Pernis (9) have reported amino acid analyses of serum protein fractions separated by electrophoresis which do not indicate any obvious relationships among the amino acids. The cause of the discrepancies between the results of Schultz *et al.* (15) and those of Pernis (9) and of Cummings and Flynn (5) is not apparent, as all three groups of investigators used essentially the same methods.

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Note

BROM CRESOL PURPLE AS AN AID IN THE LOCATION OF AMINO ACIDS ON DESCENDING PAPER CHROMATOGRAMS[1]

RICHARD H. MANDL

Since it is often desirable to allow the solvent to drip off the paper during chromatography, a marker is required to prevent elution of faster moving substances (see Block *et al.*,[2] p. 110). Brom cresol purple (dibromo-*o*-cresol-sulfonephthalein) has been found to be suitable as a marker for amino acid chromatography since it moves approximately 10 per cent faster than the leucines in the following solvents:

a) *n*-butyl alcohol—acetic acid—water (450:50:125 v/v)
b) *tert.*-butyl alcohol—formic acid—water (14:3:3 v/v)
c) 88% phenol—water (5:1 v/v)
d) 88% phenol—0.01% ammonia (5:1 v/v)
e) 2-butyl alcohol—1.5N ammonium hydroxide (3:1 v/v)
f) ethanol—*tert.*-butyl alcohol—58% ammonium hydroxide—water (60:20:5:15 v/v).

A small spot (1–10 μl.) of a 0.1 per cent ethanolic solution of brom cresol purple is applied to the base line at both ends of the paper. When a two-dimensional system is employed, a second application of the indicator solution is necessary after development with the first solvent. As the chromatogram develops, the indicator is always visible. The progress of the development can, therefore, be checked at any time.

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[1] Aided by grant A-1233, National Institutes of Health, United States Public Health Service, Bethesda, Maryland.

[2] Block, Richard J., Emmett L. Durrum, and Gunter Zweig. A manual of paper chromatography and paper electrophoresis. 2nd ed. 710 pp. Academic Press, Inc., New York. 1958.

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MECHANISM OF ACTION OF THE FUNGICIDE DICHLONE (2,3-DICHLORO-1,4-NAPHTHOQUINONE)[1]

Robert G. Owens and Helen M. Novotny

SUMMARY

Dichlone was found to accelerate endogenous respiration of *Neurospora sitophila* conidia in much the same manner as the classic phosphorylation uncoupling agent, 2,4-dinitrophenol. Comparisons between effects of dichlone and 2,4-dinitrophenol on phosphate levels show that both toxicants effectively prevent phosphorylation in proportion to inhibition of spore germination. Neither compound affected the amounts of endogenous adenosine di- and triphosphates.

Oxidation of glucose, acetate, lactate, pyruvate and α-ketoglutarate was inhibited at the same concentrations of dichlone that affected spore viability. Glycerol oxidation was accelerated, while alcohol and terminal oxidation systems were not affected.

In untreated spores the amounts of carbohydrate remained constant or increased during aerobic incubation of the spores, but decreased as much as 90 per cent when the spores were poisoned with dichlone. Much of the decrease was accounted for by increase in CO_2 formation. This shows that catabolism of carbohydrates proceeded, which is in accord with the indications of catabolism provided by experiments on respiration and phosphorylation.

Amino acids also decreased 80 to 90 per cent in poisoned spores with an attendant release of ammonia. The deamination products did not accumulate in untreated spores, but, in poisoned spores, large amounts of pyruvic, α-ketoglutaric and two unidentified keto acids were found, showing that the pathways of degradation of certain keto acids, probably derived from amino acids, were blocked.

Experiments on the synthesis of citrate, succinate and malate from acetate show that citrate synthesis is blocked by dichlone at concentrations that inhibit germination. This is in accord with respiratory data which show that acetate is not oxidized in dichlone-poisoned spores. These data along with those showing inhibition of pyruvate and α-ketoglutarate oxidase systems indicate that coenzyme A is inactivated, since it is the only factor common to each of these pathways. This was circumstantially confirmed by experiments which demonstrate the reaction of coenzyme A with dichlone *in vitro*.

The data show that the toxicity of dichlone is due to concomitant inhibition of phosphorylation, certain dehydrogenases and carboxylases and inactivation of coenzyme A. This involves many loci distributed throughout practically all major metabolic pathways, precluding effective metabolic by-passes and growth.

INTRODUCTION

Many of the data pertaining to the fungicidal mechanism of dichlone (2,3-dichloro-1,4-naphthoquinone) and other quinones were reviewed by McNew and Burchfield in 1951 (9). More recent studies show that enzymes with functional free sulfhydryl and amino groups, such as malt and pancreatic amylases, respectively (6, 7, 20, 21), are inhibited by as

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[1] This work was supported in part by a grant from the Rockefeller Foundation.

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little as $5 \times 10^{-6} M$ of dichlone *in vitro* (11). This suggests a potential relationship between the toxicity of dichlone and inhibition of enzymes and coenzymes with essential free sulfhydryl and amino groups *in vivo*. Previous experiments on respiration and phosphorylation have partially affirmed this (14).

The present study is an extension of previous work (14, 15). Its purpose is to provide a comprehensive appraisal of the effects of dichlone on metabolism of fungus spores and to correlate them with effects on viability.

MATERIALS AND METHODS[2]

Conidia of *Neuropora sitophila* (Mont.) Shear & Dodge were used for all tests. Methods of culture, harvesting conidia and determination of dry weight of the conidia were described previously (16).

Respirometry was carried out at 30° C. in the Warburg apparatus as described by Umbreit *et al.* (18). For endogenous respiration determinations, each Warburg flask contained 30 to 40 mg. dry weight of spores and the desired amount of chemical in 2 ml. of water. For experiments with exogenous substrates 30 to 40 mg. dry weight of spores, 5 mg. of substrate and the desired amount of chemical in a total of 2.5 ml. of water were used. When appropriate, the center well contained 0.2 ml. of 10 per cent KOH. The respiratory quotient (R.Q.) was calculated from values for O_2 uptake and CO_2 release obtained during the first 30 minutes of the experiments by Warburg's direct method (18, p. 28). Q_{O_2} and Q_{CO_2} values were also based on the first 30 minutes.

For other tests, spores were incubated in 125 or 250 ml. Erlenmeyer flasks on an Atlab[3] shaker for about 16 hours. The systems contained approximately 250 mg. dry weight of spores, 100 mg. of substrate and the desired amount of toxicant in 30 ml. of water. Substrate and/or toxicant was omitted in the controls. For assays on certain components, such as amino acids and other organic acids, the amounts of all components of the incubation systems were doubled.

After incubation, a drop of spore suspension was transferred from each flask to 50 ml. of distilled water. A drop of the diluted suspension was then placed on a microscope slide and mixed with one drop of Fries solution as modified by Ryan *et al.* (17). The slides were incubated in moisture chambers at room temperature until the controls were fully germinated. Counts were made as recommended by the Committee on Standardization of Fungicidal Tests of The American Phytopathological Society (1). Quadruplicate samples were counted for each treatment.

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[2] Symbols and abbreviations used in this paper are: $Q_{O_2} = \mu l. O_2/hr. \times mg.\ dry\ wt.^{-1}$; $Q_{CO_2} = \mu l./CO_2 \times mg.\ dry\ wt.^{-1}$; R.Q. = respiratory quotient; DNP = 2,4-dinitrophenol; ATP = adenosine triphosphate; ADP = adenosine diphosphate.

[3] Distributed by Arthur H. Thomas Company, Philadelphia, Pa.

The remainder of the spores were collected by centrifugation. The supernatant solution was decanted and kept under refrigeration. Various constituents were then extracted from the spores with appropriate solvents and quantitated as described previously (16). Except for inorganic phosphate determinations, spore extracts and incubation solutions were combined so that differences due to leaching of cell contents were not a factor in comparisons of treatments. For inorganic phosphate, separate assays were conducted on the supernatant solutions and on trichloroacetic acid extracts. Ammonia was determined as described by Vickery and Meiss (19).

A special incubation system was devised for carbon balance experiments. It consisted of an air inlet through three 300-ml. traps containing 10 per cent KOH to remove CO_2 from ingressing air. These were connected to duplicate samples in series on the shaker. Egressing air from the samples was then passed through three traps in series, each containing 40 ml. of 75 per cent saturated $Ba(OH)_2$ solution. The flow of air was maintained by a connection to the laboratory vacuum line and was regulated with screw clamps. The $BaCO_3$ was collected on a tared sintered glass funnel and washed with boiled distilled water until all of the $Ba(OH)_2$ was removed. The samples were dried overnight in an oven at 100° C. and weighed. Lipids, carbohydrates and bulk protein of the spores and supernatant solution were then determined (16).

Dichlone was crystallized from a preparation of Phygon.[4] Hot benzene was saturated with Phygon and filtered while still hot. The filtrate was cooled and the crystals that separated were redissolved in a minimum of hot benzene. A small amount of Norit A was added and the solution was filtered again and permitted to cool. The final crystals melted at 196–197° C. (uncorrected). The 2,4-dinitrophenol (DNP) was a commercial product melting at 112–113° C.

RESULTS

EFFECTS OF DICHLONE AND 2,4-DINITROPHENOL ON RESPIRATION

Endogenous respiration was reported previously to be accelerated by dichlone (15). This abnormally high respiratory rate is typical of effects produced by DNP and certain other nitrophenols, which at low concentrations, uncouple phosphorylation from oxidations (3, 8). Thus DNP was tested along with dichlone so that direct comparisons between effects of the two compounds could be made. It was found that both toxicants at very small dosages accelerated O_2 uptake and CO_2 release (Fig. 1). Acceleration ranged from about 10 to 30 per cent for O_2 uptake (Fig. 1 A)

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[4] Trade name under which dichlone is sold by United States Rubber Company.

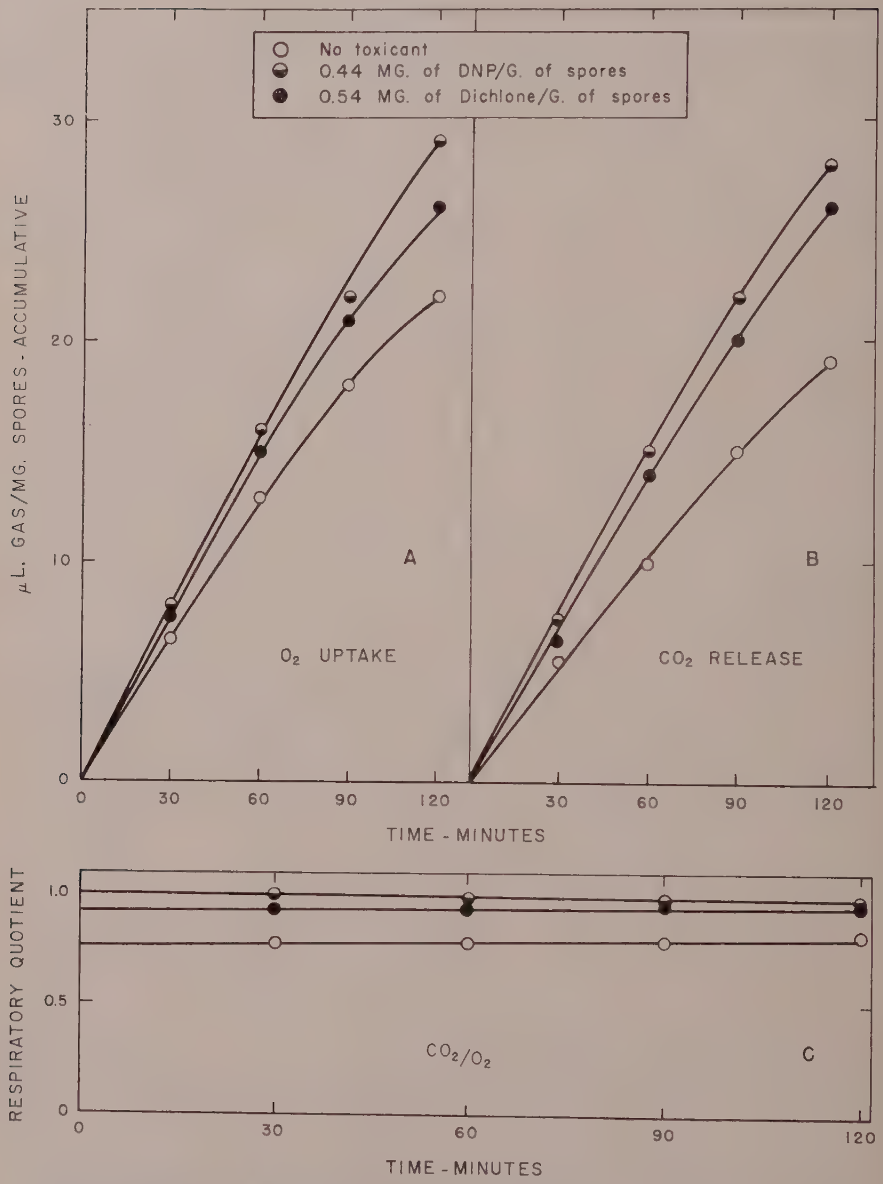


FIGURE 1. Effects of dichlone and 2,4-dinitrophenol at $5 \times 10^{-5} M$ on (A) O_2 uptake, (B) CO_2 release, and (C) the respiratory quotient of conidia of *Neurospora sitophila* in relation to time.

and from 25 to 50 per cent for CO_2 release (Fig. 1 B). While the acceleration was small in 30 minutes it was consistent from test to test and became greater over longer periods of time. Both toxicants increased the rate of CO_2 release more than that of O_2 uptake. This resulted in an increase in the R.Q. (Fig. 1 C).

At higher dosages of the order of 25 to 50 mg. of toxicant/gram dry wt. of spores, DNP inhibited respiration almost completely. Dichlone, however, accelerated respiration even at a lethal dose of 27 mg./gram (Table I). CO_2 formation was more than doubled and the R.Q. was increased to 1.32. The fact that dichlone did not inhibit respiration at the high dosage might have been due to its limited solubility, e.g., about 7 p.p.m. or $3 \times 10^{-5} M$ in water. The effective dose available probably would not exceed this amount at any given instance although large excesses of undissolved dichlone were present.

TABLE I

EFFECT OF DICHLONE ON RESPIRATION OF CONIDIA OF NEUROSPORA SITOPHILA IN THE PRESENCE OF GLUCOSE AND ACETATE*

Substrate	Dichlone	Q_{O_2}	Q_{CO_2}	R. Q.
None	—	10.8	7.8	0.72
None	+	12.5	16.5	1.32
Glucose	—	23.0	20.9	0.91
Glucose	+	11.5	20.2	1.75
Acetate	—	22.1	9.5	0.43
Acetate	+	8.3	9.8	1.18

* Dichlone was tested at a lethal dose of 27 mg./gram dry wt. of spores. Five mg. (135 mg./gram of spores) of glucose or acetate were used in each Warburg flask.

Exogenous respiration. The effects of dichlone on oxidation of a number of substrates by intact conidia were determined. Results with glucose and acetate are given in Table I. Both substrates more than doubled the Q_{O_2} in the absence of dichlone, but neither increased O_2 uptake when a lethal dose of the fungicide was present.

The Q_{CO_2} of untreated spores was affected differently by the two substrates. Glucose more than doubled CO_2 release and increased the R. Q. to .91. Acetate caused only a small increase in CO_2 formation, which, because of a relatively large increase in O_2 uptake, resulted in a decrease in the R.Q. These results are in accord with previous respiratory studies with glucose and acetate (12).

In the presence of dichlone, CO_2 formation due to glucose was not inhibited although O_2 uptake was reduced to approximately the level of the controls. CO_2 formation from acetate, however, was strongly inhibited, and the total CO_2 produced was about equivalent to that in the controls.

Other experiments, data from which are not given here, indicate that

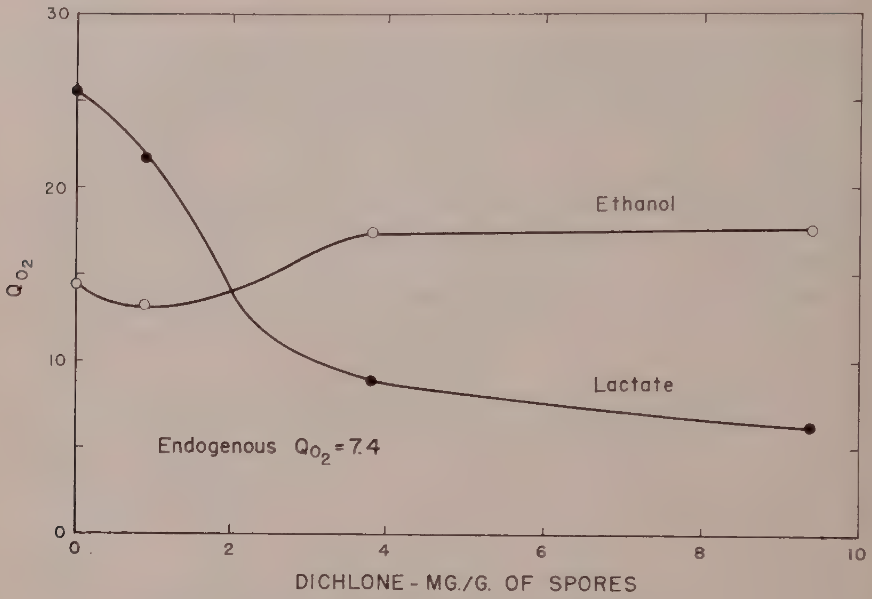


FIGURE 2. Effects of dichlone on oxidation of ethanol and lactate by conidia of *Neurospora sitophila*.

a number of electron transport systems are present in spores which form *internal* oxidation-reduction systems. For example, it was found that glucose-1-phosphate was rapidly reduced by reduced triphosphopyridine nucleotide in spore homogenates. Thus the electrons from the reduced coenzyme were transferred to an internal acceptor instead of to molecular oxygen. This type of system, which was found to be quite insensitive to dichlone, might help to account for the continued release of CO_2 without simultaneous uptake of O_2, provided the dehydrogenases responsible for the initial extraction of electrons from carbohydrates of various kinds were not inactivated. Further evidence of the presence of dichlone-insensitive dehydrogenases and/or oxidases is given in subsequent sections.

The effects of dichlone on oxidation of ethanol and lactate are shown in Figure 2. Lactate oxidation was almost completely inhibited by the fungicide at concentrations that affected germination. Oxidation of ethanol was not affected.

Glycerol was found to be oxidized relatively slowly (Fig. 3 A) compared to glucose or acetate (Table I). Addition of dichlone to systems containing glycerol, however, resulted in marked acceleration instead of inhibition of O_2 uptake in short term experiments (Fig. 3 A and B). At a lethal dose of 7.3 mg. of dichlone/gram of spores, the Q_{O_2} was more than doubled after 20 minutes. After longer periods of time it dropped slightly below

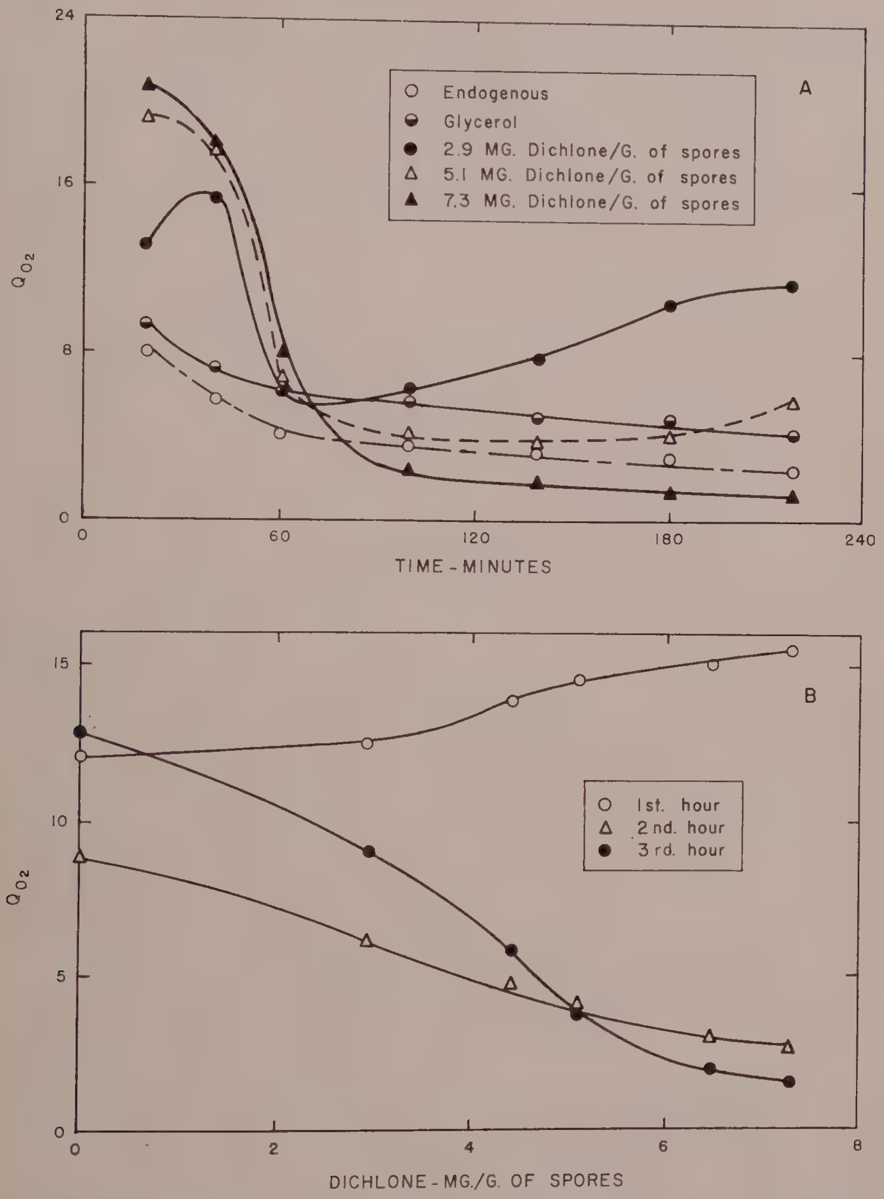


Figure 3. Effects of dichlone on O_2 uptake by conidia of *Neurospora sitophila* with glycerol at 400 mg./gram of spores as a substrate. (A) O_2 uptake in relation to time. (B) O_2 uptake in relation to the concentration of dichlone.

the endogenous Q_{O_2}. At sublethal doses (2.9 mg./gram or less), the initial acceleration was smaller than at high dosages, but after a drop during the first hour, the Q_{O_2} was almost tripled after 3 hours (Fig. 3 A).

All concentrations of dichlone tested caused an initial increase in O_2 uptake with glycerol as a substrate (Fig. 3 B), but after the first hour oxygen consumption dropped more or less in accord with the amount of dichlone present. It should be noted that during the first 40 minutes at 2.9 mg./gram the acceleration increased considerably in relation to the endogenous rate. This increase was noted above in connection with the acceleration of endogenous O_2 uptake by very small amounts of dichlone and DNP (Fig. 1). The similarities between effects on endogenous and glycerol oxidation suggest that acceleration of endogenous O_2 uptake might be related to metabolism of endogenous glycerol. Since the fungus was cultured on a medium containing glycerol as the principal carbon source, the relationship might logically be expected.

EFFECTS OF DICHLONE ON ENDOGENOUS PHOSPHATE METABOLISM

In preliminary experiments inorganic phosphate was found to increase in spores upon incubating them aerobically without substrate. Simultaneously, phosphorus in lipid and ribose nucleic acid fractions decreased (Table II). No change or slight increases occurred in the deoxyribose nucleic acid and residue fractions. This indicates that most, or perhaps all, of the increase in inorganic phosphorus was derived from dissimilation of ribose nucleic acid, phospholipids and probably phosphorylated carbohydrates under starvation conditions.

When glycerol was supplied as a substrate, the inorganic phosphate level remained constant or decreased slightly during incubation (Table III). It is assumed, therefore, that glycerol, or its metabolic products, was able to provide suitable phosphate acceptors to prevent the accumulation

TABLE II

EFFECT OF DICHLONE ON PHOSPHORUS LEVELS IN VARIOUS FRACTIONS OF NEUROSPORA SITOPHILA CONIDIA*

Treatment of spores		Phosphorus content, mg./g. spores						Germination, per cent
Incubation period	Amount of dichlone, mg./g. spores	2% Perchloric acid extract	Lipids	Ribose nucleic acid	Deoxyribose nucleic acid	Residue	Total	
None	0	2.5	3.1	15.8	2.2	1.5	25.1	100
16 hours	0	3.2	2.6	15.4	2.1	1.4	24.7	100
16 hours	3.80	3.3	2.2	15.8	2.5	1.9	25.7	85
16 hours	4.75	3.5	1.9	14.7	2.4	1.9	24.4	63
16 hours	5.75	4.2	2.2	13.8	2.2	1.9	24.3	37
16 hours	7.65	5.2	2.1	12.1	2.3	2.0	23.7	0

* Spores were incubated aerobically without addition of nutrients.

of phosphorus in the inorganic form. However, when lethal amounts of dichlone or DNP were added in the presence of glycerol, the amount of phosphorus in the inorganic state increased even more than in the incubated controls without glycerol (Tables II and III). Thus, the toxicants not only inhibited phosphorylation of glycerol and its metabolic products, but prevented reincorporation of an additional portion derived from organic phosphates. The amounts of adenosine diphosphate (ADP) and adenosine triphosphate (ATP) were not affected by either toxicant (Table III).

Inhibition of phosphorylation, as indicated by accumulation of inorganic phosphate, was found to be approximately proportional to inhibition of germination over a range of dichlone concentrations producing 0 to 100 per cent loss of viability (Fig. 4).

It was found also that dichlone and DNP caused release of a considerable amount of inorganic phosphate into the incubation medium (Table IV). The significance of this in the mode of action of dichlone is dubious, however, since large amounts of many kinds of cell constituents can be leached from spores without loss of viability (2, 10).

Although the mechanism by which phosphorylation is uncoupled from oxidation is not fully understood, DNP is known to dissociate synthesis of ATP from oxidation (3, 8). In the present experiments neither ATP nor ADP levels were affected by dichlone or DNP (Table III). It would appear, therefore, that the polyphosphates were not directly involved in the accumulation of inorganic phosphate. If it is assumed that DNP dissociated synthesis of ATP from oxidation in the spores, it follows that ATP utilization and breakdown were inhibited. Whether this was the result of inhibition of kinases which normally function in phosphate transfer or of

TABLE III

EFFECT OF DICHLONE AND 2,4-DINITROPHENOL AT LETHAL DOSES ON THE AMOUNTS OF INORGANIC PHOSPHATE, ADENOSINE DIPHOSPHATE (ADP) AND ADENOSINE TRIPHOSPHATE (ATP) IN CONIDIA OF NEUROSPORA SITOPHILA WITH GLYCEROL* AS A SUBSTRATE

Treatment of spores			Phosphate, mg./g. of spores				Percentage change	
Incubation period	Toxicant							
	Name	Amount, mg./g. of spores	Inorganic	ADP	ATP	ADP + ATP	Inorganic phosphate	ADP + ATP
None	None	0	3.01	0.89	1.18	2.07	—	—
16 hours	None	0	3.15	1.08	1.12	2.20	+ 4	+6
16 hours	2,4-Dinitrophenol	7.5	4.35	0.95	1.02	1.97	+31	−5
16 hours	Dichlone	9.1	3.87	0.94	1.08	2.02	+22	−4

* The glycerol concentration was 400 mg./g. of spores in 30 ml. of water.

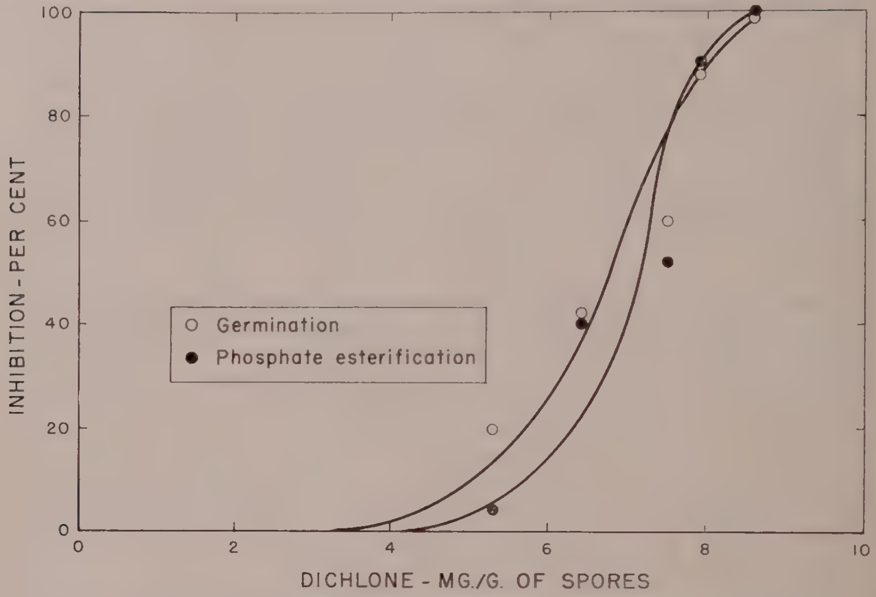


FIGURE 4. Effects of dichlone at various concentrations on germination and on phosphate esterification by conidia of *Neurospora sitophila*.

blocks in pathways preceding or following phosphate transfer is not clear. Evidence presented in a subsequent section concerning inactivation of coenzyme A, however, suggests that certain reactions involving phosphate transfer may be blocked at points following phosphorylation. In this event the whole chain of interconversions would become static and ATP utilization would be inhibited as effectively as if the kinase concerned with phosphate transfer were inhibited.

TABLE IV

EFFECT OF DICHLONE AND 2,4-DINITROPHENOL AT LETHAL DOSES ON LEVELS OF INORGANIC PHOSPHATE IN CONIDIA OF NEUROSPORA SITOPHILA AND IN THE AMBIENT SOLUTION AFTER AEROBIC INCUBATION*

Treatment of spores			Inorganic phosphate, mg.		
Incubation period	Toxicant		Ambient solution	Trichloro-acetic acid extract	Total
	Name	Amount, mg./g. of spores			
None	None	0	—	1.25	1.25
16 hours	None	0	0.28	1.17	1.45
16 hours	2,4-Dinitrophenol	7.5	1.08	1.23	2.31
16 hours	Dichlone	9.1	0.99	1.06	2.05

* Spores were incubated without added substrate.

EFFECTS OF DICHLONE ON CARBOHYDRATE METABOLISM

Experiments on respiration show that some phases of metabolism resulting in CO_2 release proceeded in the presence of lethal doses of dichlone (Table I and Fig. 1). The decreases in lipid and ribose nucleic acid (Table II) were not sufficient to account for a sustained release of CO_2 of the magnitude found. Consequently, a study of the carbon balance between CO_2, carbohydrates, and other cell components was made. Untreated spores incubated aerobically for 16 hours released more than 20 per cent of their dry weight as CO_2 (Table V). Most, if not all, of this was accounted for by decreases in lipids and proteins. The amount of carbohydrate increased about 20 per cent.

TABLE V

Effect of Dichlone at a Lethal Dose on the Amounts of CO_2 Released, and on the Carbohydrate, Lipids, Protein and Other Components of Neurospora sitophila Conidia Incubated Aerobically with and without Acetate as a Substrate

Treatment	Substrate**	CO_2 released, mg./g. of spores	Spore constituents, mg./g.				Total spore constituents assayed + CO_2 mg./g.	Change from untreated control, per cent
			Carbohydrate	Lipids	Protein	Residue		
None	None	222	114	46	113	320	815	—
Dichlone*	None	322	14	46	105	315	802	− 2
None	Acetate	408	120	41	74	418	1061	+33
Dichlone*	Acetate	339	11	46	72	347	815	0

* 9.9 mg. of dichlone/g. of spores.
** Approximately 400 mg. of sodium acetate/g. of spores.

In the presence of lethal doses of dichlone the amount of CO_2 released increased to more than 32 per cent of the dry weight of the spores. The decrease in lipids and protein was almost identical to that in the untreated controls but the carbohydrate level dropped about 90 per cent. The decrease in carbohydrate, therefore, accounts for a large part of the increase in observed CO_2.

A similar carbon balance study was made with acetate as a substrate (Table V). The data show that CO_2 release by untreated spores was almost doubled in the presence of acetate. The amounts of carbohydrate and lipid remaining were equivalent to those of the controls without acetate. Protein decreased slightly, but the data indicate that most of the additional CO_2 resulted from dissimilation of acetate and its metabolic products. Other studies with C^{14}-labeled acetate, not reported here, confirm this.

In the presence of lethal amounts of dichlone, acetate did not increase CO_2 release and did not prevent dissimilation of carbohydrate. This is in accord with the inhibition of acetate oxidation by dichlone shown above in respiratory studies (Table I). Protein content decreased slightly more in the presence of acetate than in the dichlone-treated control without acetate.

Data in Table I show that although oxidation of glucose was inhibited by lethal doses of dichlone CO_2 release was not affected. This suggests that glucose was either dissimilated anaerobically or via the dichlone-insensitive aerobic pathway through which endogenous respiration proceeds. To determine which of these possibilities was the case, spores were incubated anaerobically for 16 hours in fermentation tubes with lethal amounts of dichlone. Under these conditions no CO_2 was produced. This shows that carbohydrate dissimilation was an oxidative process even though the primary glucose oxidase system was inhibited by dichlone. It also establishes that at least two pathways for degradation of glucose, and probably other carbohydrates, exist in the spores and that only one of them is sensitive to dichlone.

EFFECTS OF DICHLONE ON METABOLISM OF AMINO AND OTHER ORGANIC ACIDS

Free amino acids and amides decreased about 80 per cent in untreated spores during aerobic incubation for 16 hours (Table VI). A similar decrease occurred in the presence of lethal amounts of dichlone. In general the disappearance of individual amino acids and amides was about equal in treated and untreated spores, so that no particular effect of dichlone on metabolism of the individual amino acids was apparent.

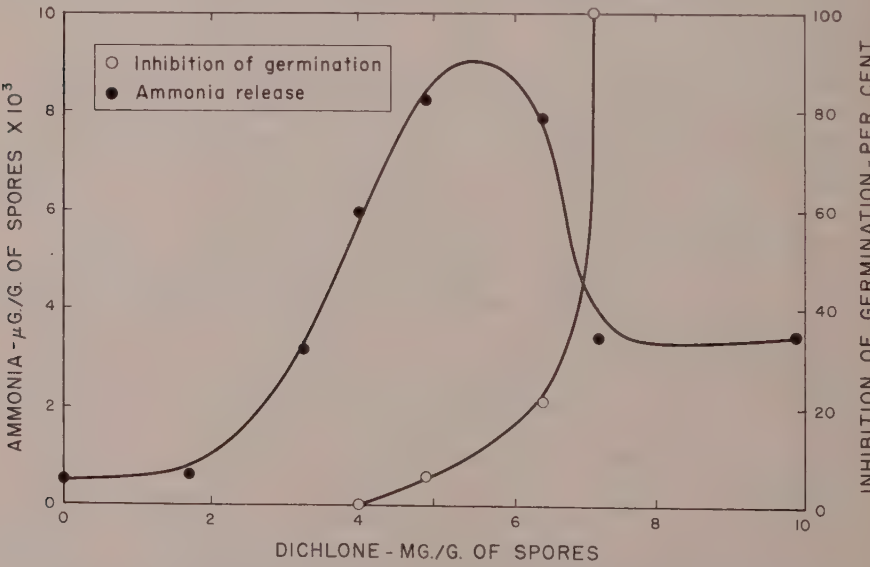


FIGURE 5. Effects of dichlone at various concentrations on germination and on release of ammonia by conidia of *Neurospora sitophila*.

TABLE VI

EFFECT OF DICHLONE AT A LETHAL DOSE ON AMOUNTS OF AMINO ACIDS AND AMIDES IN CONIDIA OF NEUROSPORA SITOPHILA AFTER INCUBATION FOR 16 HOURS UNDER AEROBIC CONDITIONS

amino acid or Amide	Treatment				
	None	Incubated without additives		Incubated with dichlone*	
	µg./g. of spores	µg./g. of spores	Decrease, %	µg./g. of spores	Decrease, %
Cysteic acid	150	20	87	5	97
Aspartic acid	60	20	67	25	58
Glutamic acid	575	195	66	35	94
Serine	270	30	89	70	74
Glycine	185	15	92	50	73
Asparagine	485	200	59	235	51
Threonine	300	10	97	20	93
Alanine	1845	255	86	160	91
Glutamine	1125	105	91	195	83
Lysine	205	5	98	5	98
Arginine	169	110	35	110	35
Proline	50	0	100	0	100
Valine	330	25	92	25	92
Leucines	575	40	93	70	88
Phenylalanine	250	25	90	55	78
Tyrosine	215	25	88	40	81
γ-Aminobutyric acid	710	15	98	110	84
Methionine sulfoxide	440	85	81	40	91
Histidine	265	85	68	70	74
Total	8204	1265		1320	
Average % decrease	—		83		81

* 9.5 mg./g. of spores.

Dissimilation of amino acids and amides was accompanied by release of ammonia. Recovery of ammonia plotted against the concentration of dichlone produced an unusual curve with an inversion near the point for 5 mg. of dichlone per gram of spores (Fig. 5). This suggests either that deamination of amino acids and amides was inhibited by dichlone at high concentrations, or that some of the ammonia was lost. The amino acid data (Table VI) indicate that dichlone did not inhibit deamination even at 9.5 mg./gram of spores. Therefore, it is assumed that some of the ammonia was lost during incubation. This implies that the ammonia-binding capacity of the spores and incubation medium was somehow reduced at high concentrations of dichlone or that ammonia reacted with dichlone.

Results of subsequent keto acid determinations suggest that the former assumption might be true. It was found that pyruvic, α-ketoglutaric and two unidentified keto acids accumulated in relatively large amounts in poisoned spores, but not in untreated spores (Table VII). These acids totaled more than 3 mg./gram of spores at 7.2 mg. of dichlone/gram. This

TABLE VII

EFFECT OF DICHLONE ON CONCENTRATIONS OF KETO ACID IN CONIDIA OF *NEUROSPORA SITOPHILA* INCUBATED AEROBICALLY FOR 16 HOURS WITHOUT NUTRIENTS

Amount of dichlone, mg./g. of spores	Keto acid,* µg./g. of spores						
	Oxalo-succinate	α-Keto-glutarate	Pyruvate	Glyox-ylate	U_1**	U_2**	Total
0	1	30	25	4	0	0	60
1.8	12	475	360	0	25	175	1047
3.6	12	540	840	0	70	180	1642
4.5	15	575	1400	0	85	215	2290
5.4	23	540	1600	0	130	230	2523
7.2	13	320	2175	0	395	240	3143
9.1	13	100	480	0	405	490	1488

* As the 2,4-dinitrophenylhydrazone.
** Unidentified acids.

undoubtedly lowered the pH of the medium sufficiently to bind the quantities of ammonia recovered. However, at higher concentrations of dichlone, the amounts of keto acids dropped, resulting in an inversion in the curve (Fig. 6) similar to that noted for the ammonia curve (Fig. 5).

The data in Table VII show that keto acids did not accumulate during incubation of untreated spores although amino acids disappeared. This indicates that dissimilation of amino acids proceeds beyond the deamination step. Since no other amino acid dissimilation products were found it

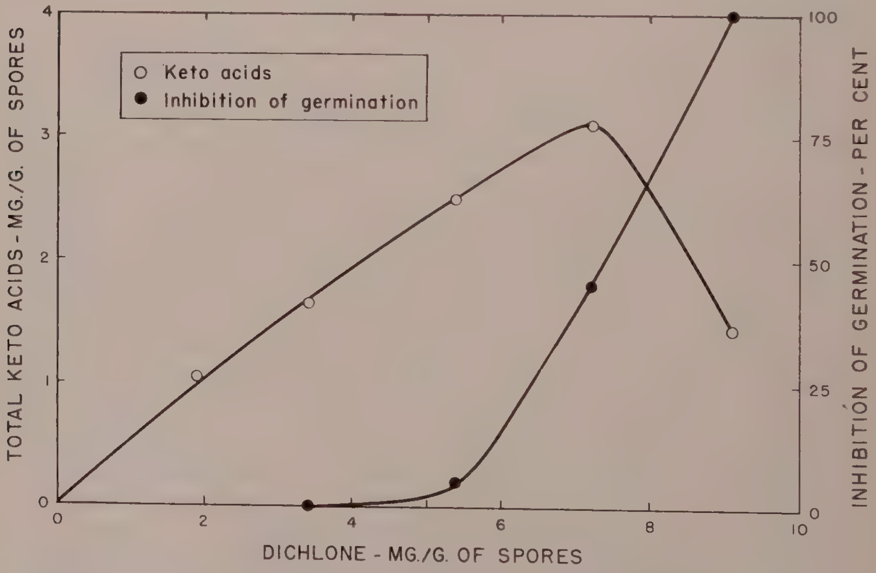


FIGURE 6. Effects of dichlone at various concentrations on the total amount of keto acids and on germination of conidia of *Neurospora sitophila*.

TABLE VIII

EFFECT OF DICHLONE AT A LETHAL DOSE ON THE AMOUNTS OF NONKETO ACIDS IN CONIDIA OF NEUROSPORA SITOPHILA AFTER AEROBIC INCUBATION FOR 16 HOURS WITH ACETATE

Substrate*	Dichlone, mg./g. of spores	Acid, mg./g. of spores		
		Succinate	Malate	Citrate
None	0	0.34	0.14	0.81
Acetate	0	0.84	0.74	2.12
Acetate	10.3	0.16	0.20	0.66

* The concentration of acetate was approximately 400 mg./g. of spores.

is probable that they were degraded to CO_2 and water. Accumulation of keto acids apparently derived from amino acids in dichlone-poisoned spores, however, indicates that certain keto acid oxidation and decarboxylation reactions were inhibited.

Since the oxidative degradation of pyruvic and a number of other keto acids involves coenzyme A and lipoic acid, further experiments on organic acid metabolism were conducted to determine whether these cofactors were inactivated by dichlone. Untreated spores incubated with acetate showed substantial increases in citrate, succinate and malate (Table VIII). Experiments with C^{14}-labeled acetate showed that these acids were derived directly from acetate, apparently by way of the conventional Krebs cycle. However, when the spores were incubated with acetate in the presence of lethal amounts of dichlone, no increase in the acids occurred. This indicates that the three-step pathway from acetate to citrate was blocked. Since lipoic acid does not participate in this pathway, it appears that coenzyme A was the factor involved both in this reaction and in the oxidation of keto acids. Further experiments with dichlone at various concentrations show that germination and citrate synthesis from acetate were affected concomitantly by the fungicide and that the magnitude of the effects was closely parallel (Fig. 7).

REACTION OF COENZYME A WITH DICHLONE

The block in synthesis of citrate from acetate, along with inhibition of keto acid metabolism (Table VII), strongly suggests that coenzyme A was inactivated, because it is the only factor known to be common to both reactions. Consequently, attempts were made to assay coenzyme A directly by the method of Lipmann (5). Results were uniformly unsatisfactory, perhaps because sulfanilamide was not readily absorbed by the spores. Spore homogenates showed no citrogenase activity, which is in accord with previously reported failures to obtain active pyruvate and acetate oxidase systems in spore homogenates (13).

Subsequently, a saturated aqueous solution of dichlone was prepared and mixed directly with 2 mg. of coenzyme A in 10 ml. of water. This re-

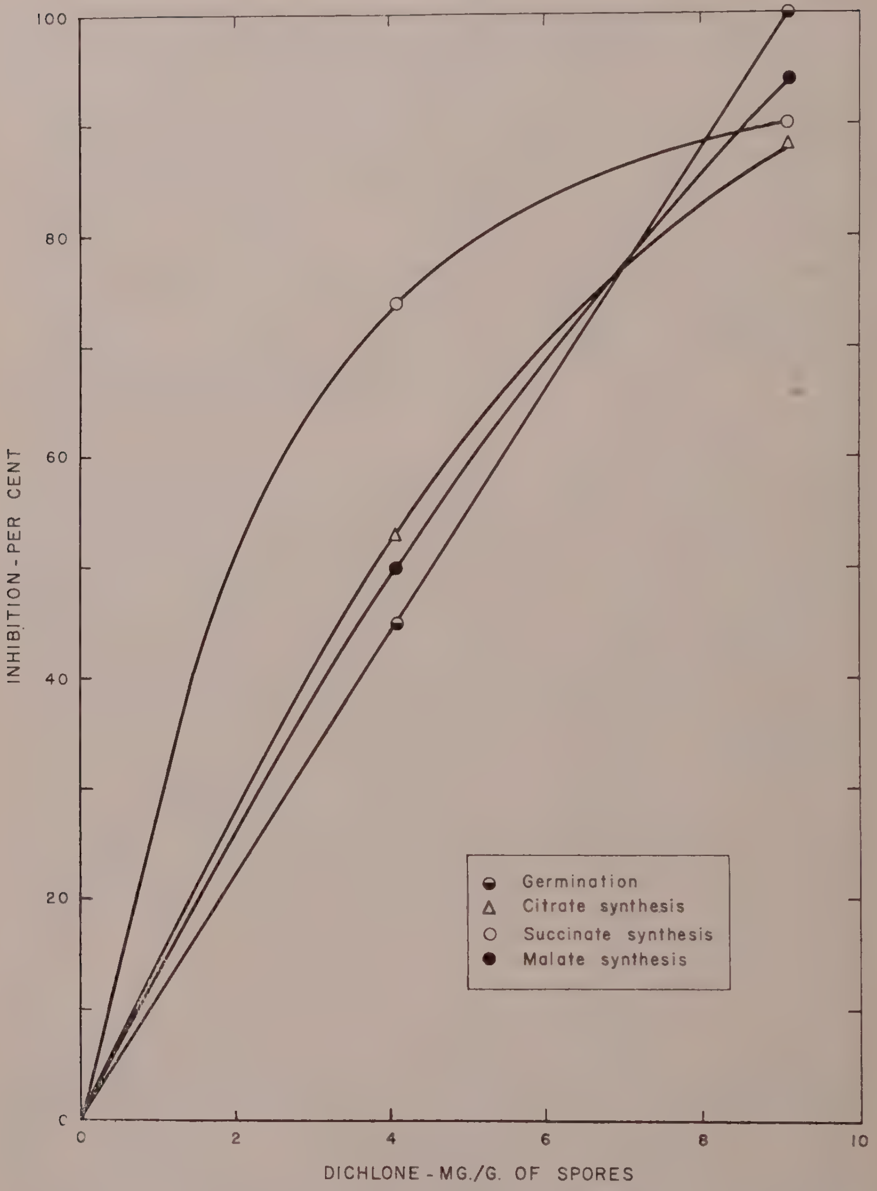


FIGURE 7. Effects of dichlone at various concentrations on germination and on the synthesis of citrate, succinate and malate by conidia of *Neurospora sitophila*. Spores were incubated 16 hours with 400 mg. of anhydrous sodium acetate/g. of spores.

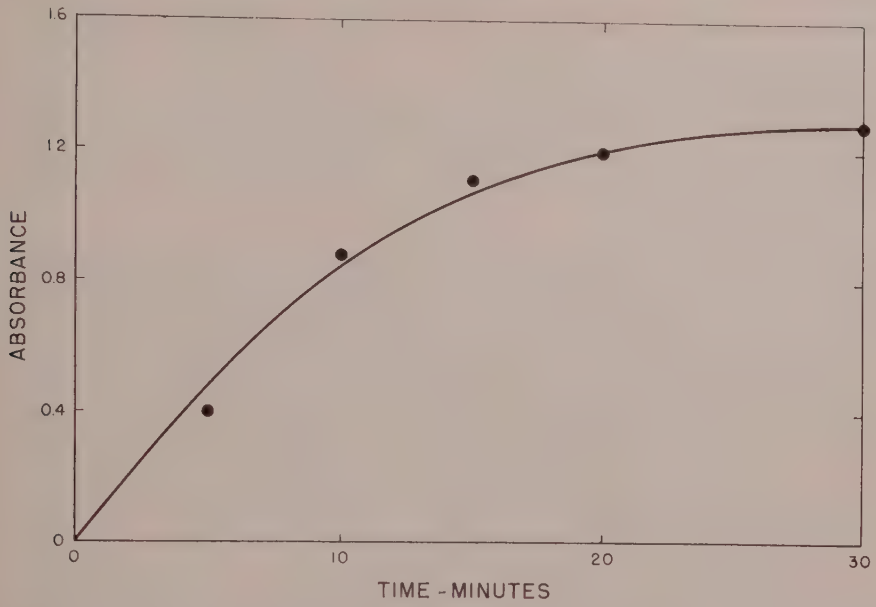


Figure 8. Rate of reaction of dichlone and coenzyme A in unbuffered aqueous solution at room temperature. Absorbance was determined at 448 mμ.

sulted in formation of a brown reaction product with an absorption maximum at 448 mμ. By measuring the rate of formation of the reaction product, it was found that the reaction was essentially complete in 15 to 20 minutes under the conditions of the experiment (Fig. 8). This circumstantially supports other indications that coenzyme A was inactivated by dichlone in the spores.

DISCUSSION

The data presented here show that important changes in spore metabolism are induced by dichlone. The first observed was a large increase in R.Q. due to acceleration of CO_2 formation from endogenous substrates. Other experiments indicate that lipids and protein are the principal sources of the carbon released as CO_2 over a period of 16 hours in untreated spores. Complete oxidation of these components theoretically would result in an R.Q. of 0.8 to 0.9, depending upon the relative amounts of each oxidized. An observed R.Q. of 0.72 shows that a portion of the oxygen is utilized without formation of a corresponding amount of CO_2. It follows, therefore, that part of the energy derived from oxidations is conserved.

In spores poisoned with dichlone the R.Q. increased to 1.32, due to the fact that CO_2 formation was accelerated relatively much more than O_2 uptake. This denotes a fundamental change in energy relationships in which the O_2 taken up is utilized to a much greater extent in CO_2 formation than it is in untreated spores. Thus the energy of oxidation is dissipated through formation of CO_2 and water which can not be further metabolized to release chemically usable energy.

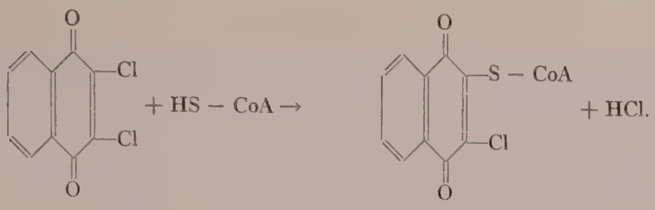
The increase in R.Q. was accompanied by near depletion of carbohydrate reserves, which in untreated spores remained constant or increased. This suggests that the conservation of energy is associated with carbohydrates or compounds which yield carbohydrates upon hydrolysis during the assay procedure.

In all living cells conservation of the energy of oxidations is accomplished through so-called "high energy" phosphates. Formation of the phosphates is coupled to the electron transport system and apparently limits the rate at which oxidation of certain substrates takes place. This is manifested by the fact that cells administered small amounts of the classic phosphorylation uncoupler, DNP, respire at higher rates than untreated cells (3, 8). Dichlone affected respiration and phosphorylation in the same manner as DNP. The evidence, therefore, suggests that the two processes are uncoupled and that the rate-limiting phosphorylation step in carbohydrate dissimilation is by-passed.

The continued or elevated uptake of O_2 by dichlone-poisoned spores reveals that the terminal oxidases are not inhibited. This is supported indirectly by the fact that no CO_2 is formed under anaerobic conditions. It reveals also that certain dehydrogenases or direct oxidases are not inhibited. This is borne out by experiments in which glycerol and alcohol were oxidized, and by the fact that carbohydrate, amino acids, protein, lipids and some of the Krebs cycle acids are oxidatively dissimilated by poisoned spores. Reactions involving these components establish clearly that not all phases of metabolism are affected by dichlone.

Other experiments, however, demonstrate that certain key oxidations are inhibited. These include oxidation of glucose, acetate, pyruvate and α-ketoglutarate. Complete oxidation of these intermediates involves a coupled system of enzmyes in the Emden-Meyerhof pathway and/or the Krebs cycle. Entrance of glucose, acetate and pyruvate residues into the cycle is mediated by citrogenase in collaboration with coenzyme A. Conversion of α-ketoglutarate to succinate also involves coenzyme A. Thus the data strongly indicate that coenzyme A is inactivated by dichlone since it is the only factor common to all of these reactions. This might well account for inhibition of oxidations of glucose and acetate which are dependent upon a chain of reactions where coenzyme A is functional in one or more steps.

Reaction of dichlone with coenzyme A is consistent with previous experiments in which dichlone was found to inhibit certain enzymes with essential, free sulfhydryl groups (11) and with known reactions of compounds containing active halogen. The reaction probably involves first a simple substitution as follows:



I II

Whether subsequent degradation of II occurs is at present unknown.

Although the evidence points to inactivation of coenzyme A as a key block in oxidation of pyruvate, it must also be recognized that anaerobic decarboxylation of pyruvate is inhibited. Since anaerobic decarboxylation proceeds without oxygen or coenzyme A, it is apparent that pyruvic carboxylase *per se* is inhibited. This is in accord with Kuhn and Bienert (4) who found that a number of quinones inhibit carboxylase.

It is concluded from the present data that the toxic effects of dichlone on fungus spores involves principally inhibition of three fundamental processes: (a) phosphorylation, (b) certain oxidations and (c) formation of acyl- or aryl-coenzyme A. Phosphorylation occurs at many steps in biological systems, so that inhibition of phosphorylation implies inactivation of enzymes at many loci in the cell. Failure in the phosphorylation system further affects all synthetic processes requiring phosphorylated intermediates for endothermic coupling of carbon fragments to form larger molecules.

Certain oxidations are essential for the release of energy and for phosphorylation, by means of which the energy is conserved. Thus in a broad sense phosphorylation and oxidation are equivalent, since both are links in a chain of reactions essential for synthesis of large molecules and, hence, for growth.

Coenzyme A is involved in practically all arylation and acylation reactions in living cells. Removal of coenzyme A by irreversible reaction with dichlone precludes all such reactions, including synthesis of proteins and lipids.

The evidence points preponderantly to concomitant inhibition of the above processes, which is closely correlated with loss of viability of the spores. It might be argued that inhibition is a result, not the cause, of inhibition of germination. This, however, belabors the negative point of view and appears to be untenable for it was demonstrated that many reactions proceed rapidly in poisoned spores. Furthermore, the inhibited reactions proceed in cell homogenates in the absence of inhibitors, which shows that destruction of cellular integrity does not necessarily preclude these processes. Growth without these processes, however, has never been demonstrated among living organisms.

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BIOCHEMICAL STUDIES OF DORMANCY AND AFTER-RIPENING IN SEEDS. I. CHANGES IN FREE AMINO ACID CONTENT

Jean MacNab Fine and Lela V. Barton

SUMMARY

The free amino acid contents of embryo and endosperm during the after-ripening of the epicotyl of tree peony at 5° C. were compared with those of the non-after-ripened seedlings in the greenhouse. Alanine and glutamine were present in greater quantities in the embryos and endosperm of seeds held at 5° C. than in those in the greenhouse. Embryos at 5° C. contained more glutamic acid and valine and endosperm material at 5° C., more serine and possibly more glycine. On the other hand, asparagine, phenylalanine and tyrosine contents of embryos were greater in the seedlings at greenhouse temperature than in those at 5° C. Amino acids which did not appear to be affected by temperature treatment of the seedlings were γ-aminobutyric acid, aspartic acid, leucine, lysine, proline and threonine; comparable increases in quantity with increased time after planting were noted in the greenhouse and at 5° C. Arginine and unidentified substance(s) were present in large quantity but were not separated by the procedures used.

The amino acid contents of seedlings treated with gibberellic acid to break epicotyl dormancy in the greenhouse without low temperature pretreatment showed trends resembling those of the greenhouse controls (nonafter-ripened seedlings) rather than those after-ripened at 5° C., except for alanine which followed the same pattern as seedlings after-ripened at the low temperature.

The possible significance of these findings in relation to the breaking of dormancy is discussed.

INTRODUCTION

It is well known that many seeds will not germinate until they have had a pretreatment period in a moist medium at a low temperature. Under these conditions after-ripening takes place, involving changes which prepare the endosperm (or stored food) as well as the embryo for the germination process. Some of these changes have been studied and reports of the breaking up and dispersal of lipoid particles, the increasing acidity, the larger quantities of amino nitrogen and reducing sugars, and the activation of enzymes are to be found in the literature (3, pp. 161–167). The dormant seeds used for these tests were of the type which require after-ripening before a root is formed.

Tree peony (*Paeonia suffruticosa* Haw.) seeds if placed in a moist medium at temperatures of about 25° C. will germinate to form roots. If left at these temperatures the root system will extend, using the food stored in the large endosperm of the seed, but few of the seedlings will produce green shoots above ground. This dormancy of the portion of the seedling which forms the shoot has been called epicotyl dormancy.

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It is known from their physiological behavior that 8 to 12 weeks at 5° C. are sufficient for complete after-ripening of tree peony epicotyls (1); also that gibberellic acid treatment replaces the need for this low temperature. Morphological changes taking place in epicotyledonary tissues have been traced at intervals of from 2 to 12 weeks in soil both in the greenhouse and at 5° C. (1). It was found from microscopic examination of sectioned material that differentiation and elongation proceed slowly in untreated seedlings in the greenhouse. More rapid development, especially elongation, of the epicotyl is induced by gibberellic acid or by a period of 8 weeks at 5° C. This elongation is characteristic of seedlings capable of green shoot production in soil in the greenhouse and does not occur in untreated seedlings which fail to produce green shoots.

The seedlings used for amino acid analyses to be given below were replicates of the lots used for the physiological and morphological studies already reported (1). Hence, any changes found in the amino acid contents can be definitely related to the after-ripening process.

MATERIALS AND METHODS

Seedlings. The tree peony seedlings were produced in the same way and at the same time as described in a previous publication (1). Seeds of the 1956 crop collected at Yonkers, N. Y., were mixed with moist granulated peat moss and held at controlled daily alternating temperature of 15° to 30° C. (16 hours at 15° C. and 8 hours at 30° C. daily for five days followed by 15° C. for two days each week). Root production under these conditions began in about six weeks. Seedlings were removed from the peat moss when the roots were 25 to 35 mm. long. After removal from the peat moss, 25 seedlings each were planted in 6-inch pots in a mixture of soil, granulated peat moss, and sand in equal parts. Pots were placed in the greenhouse for green shoot production with and without a preceding period at 5° C. Also certain seedlings were treated with gibberellic acid [in the manner previously described (1)] immediately upon removal from the peat moss and were planted in the soil mixture and placed in the greenhouse.

Preparation of extract. Extracts of the free amino acids present in the embryo and endosperm were made after 0, 2, 4, 6, 8, 10 and 12 weeks in the greenhouse or at 5° C. Also, seedlings receiving 100 μg. each of gibberellic acid, together with their endosperm portions, were extracted after 2, 4, 7, 9, 11, 14 and 22 days in the greenhouse. Dry seeds and seeds which had been in granulated peat moss for 13 weeks but had not germinated were extracted as controls for the germinated seeds used for planting in the soil. In all cases duplicate samples of 20 seedlings each from two different pots were extracted.

Seedlings to be extracted were removed from the soil and washed with distilled water. Four samples which had received no treatment other than germination in peat moss were removed from the peat moss and extracted in the same way. Seed coats were removed from the endosperm portion and discarded. The endosperm was separated from the seedling portion, designated embryo, and fresh weights for 20 endosperms and 20 embryos in duplicate were recorded.

The samples were homogenized in a VirTis homogenizer with 15 ml. of 80 per cent ethanol. The insoluble residue was filtered out with Whatman No. 1 paper and washed thoroughly with 80 per cent ethanol to give a total extract volume of 125 ml.

Each extract was then purified by passing through 5 ml. of Dowex 50-X-8, 200–400 mesh, H^+ resin bedded in 80 per cent ethanol in a 1 × 30-cm. glass tube with a fritted glass disc in the bottom. The flow rate, regulated by air pressure, was about 2 ml. per minute. The amino acids retained on the column were eluted with $0.4N$ NH_4OH in 80 per cent ethanol and $4.0N$ aqueous NH_4OH according to the method of Plaisted (10), and the resulting solution evaporated to dryness under a stream of air.

The residue was made up to 1 ml. volume with 10 per cent isopropanol and was stored at $-18°$ C.

Embryos from 20 dry or moist ungerminated seeds contained quantities of amino acids too small to be measured when aliquots up to 60 μl. were used. Further tests with the dry embryos, in which all of the extract from the 20 embryos was used, showed the presence of free amino acids, but the identity and quantity could not be determined because of the large amount of extraneous matter. Consequently, extracts were made of 600 dry embryos, and chromatograms were prepared using 20- and 40-μl. aliquots. Satisfactory quantitative measurements could then be made.

Chromatography. The quantitative determination of free amino acids was done according to the method of Porter *et al.* (11). From 5 to 40 μl. of extract, depending upon the quantity of amino acids therein, were applied to Whatman No. 3 paper, $18\frac{1}{2}$ inches × $22\frac{1}{4}$ inches. Eighty-eight per cent phenol-water (4:1 v/v) adjusted to pH 5.5 with NaOH was the solvent for the first direction. A beaker of 100 ml. of 0.5 per cent NH_4OH was placed in the tank to increase the Rf values of the basic amino acids, lysine and arginine, which were present in such large quantities as to interfere with separation of other spots. However, this caused arginine to overlap an unidentified spot so that no determinations of arginine were made in the course of this experiment.

The solvent for the second direction was *n*-butanol–acetic acid–water (90:10:29 v/v/v). The papers were dried thoroughly after removal from each solvent.

After applying 1.0 per cent ninhydrin in absolute ethanol containing 0.1 per cent 8-hydroxyquinoline to the papers, colors were developed in an ethanol-saturated atmosphere at 60° C. for 30 minutes. The colored spots were eluted in 50 per cent ethanol containing 0.025M phosphate buffer. Their absorbances were determined the following day (16 to 24 hours after elution) on a Beckman spectrophotometer. Duplicate chromatograms were prepared of each of two extracts of embryo and endosperm for each treatment.

Quantities of amino acids in the extracts were determined by comparing their absorbances with standard curves, and are expressed as micrograms per 20 seedlings in the data to be presented below. Fresh weights of both embryo and endosperm portions were taken before extraction but dry weights were not determined.

RESULTS

The free amino acid content of embryos of dry or moist ungerminated seeds was very low (Table I). It will be recalled that 600 dry embryos were extracted in order to obtain quantitative measurements, while only 20 moist ungerminated embryos were used. This accounts for lack of data for most of the amino acids of the latter. It is obvious, however, that mobilization of free amino acids in the embryos was greatly accelerated by the germination process rather than by the absorption of water before germination. On the other hand, the endosperm of dry or moist ungerminated seeds contained fairly large amounts of free amino acids (Table I).

The averages of four determinations each (two extracts and duplicates of each) of the amino acids in germinated seeds at each of the periods in the greenhouse and at 5° C. are also shown in Table I. Comparisons of greenhouse and 5° C. samples were made by a method suggested by Rao (12) using the t test applied to the coefficients obtained by this method.[1] Amino acids and amides showing statistical differences in amounts at the two temperatures have been indicated by asterisks in Table I.

AMINO ACIDS WHICH ACCUMULATE AT 5° C.

Alanine. The alanine contents of embryos and endosperm in the greenhouse and at 5° C. are shown in Table I and Figure 1. The rapid and large increase in amount of this acid at 5° C. is apparent. Starting from 242 μg. at the time the seedlings were planted in soil, the content in the embryos at 5° C. increased rapidly to double the amount within two weeks, while the increase in the greenhouse seedlings was much less marked. Concomitant with the increase in the embryo at 5° C. was a somewhat smaller, but definite increase in the endosperm. Since the endosperm, representing the stored food in the seed, must supply the needs of the embryo and since

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[1] The authors wish to express their thanks to Dr. Frank Wilcoxon who suggested methods for statistical analysis and assisted in the evaluation of the data.

TABLE I

AMINO ACID CONTENTS OF EMBRYO AND ENDOSPERM TISSUES OF GERMINATED SEEDS OF TREE PEONY PLANTED IN SOIL AND HELD IN THE GREENHOUSE OR AT 5° C. FOR 2 TO 12 WEEKS. FIGURES ARE AVERAGES OF FOUR REPLICATE DETERMINATIONS

Amino acid	Tissue	Amino acid content after germination (μg./20 seeds)										
		Dry	Moist		Germinated seeds after weeks in							
					Greenhouse				5° C. room			
			Un-germinated	Germinated	2	4	8	12	2	4	8	12
Alanine	Embryo**	14	10	242	350	318	534	605	466	482	875	1,042
	Endosperm**	1,000	1,467	655	488	259	183	222	740	855	809	942
γ-Aminobutyric acid	Embryo	6	†	94	275	425	550	971	94	208	475	849
	Endosperm	401	434	292	329	284	283	385	121	101	68	191
Asparagine	Embryo**	14	†	2,375	4,438	8,342	12,013	15,019	2,327	2,325	4,276	4,800
	Endosperm**	1,534	1,402	2,483	1,968	2,674	2,476	2,959	1,412	491	475	598
Aspartic acid	Embryo	15	30	463	900	729	1,233	1,433	632	881	1,450	1,609
	Endosperm	1,999	2,633	2,016	1,281	1,088	1,383	764	1,369	771	455	751
Glutamic acid	Embryo*	17	100	675	1,187	1,509	2,000	2,390	1,184	1,932	3,496	3,034
	Endosperm	5,335	9,533	5,569	4,633	5,017	6,599	3,464	3,320	2,431	3,249	3,645
Glutamine	Embryo**	5	†	5,738	10,966	12,800	14,130	13,488	15,273	16,575	31,433	42,850
	Endosperm*	266	266	886	1,393	976	959	1,088	2,179	1,765	1,209	1,680
Glycine	Embryo	2	†	63	125	105	168	188	84	104	208	83
	Endosperm	201	168	85	105	104	42	94	104	105	84	210
Leucine	Embryo	2	10	388	500	796	777	1,071	463	558	400	883
	Endosperm	117	301	300	138	274	157	161	112	264	392	682
Lysine	Embryo	3	†	†	350	856	1,483	1,374	†	475	975	1,174
	Endosperm	167	402	133	51	225	225	308	†	88	125	258
Phenylalanine	Embryo*	13	†	38	250	592	875	674	294	513	616	334
	Endosperm	417	567	308	267	766	701	961	329	522	642	879
Proline	Embryo	10	†	25	462	413	284	159	150	275	1,174	549
	Endosperm	383	301	175	350	138	117	410	†	63	142	193
Serine	Embryo	6	20	313	524	676	868	926	431	529	975	1,368
	Endosperm*	433	668	526	464	362	308	326	441	584	725	1,122
Threonine	Embryo	3	†	278	500	467	1,107	1,415	644	234	559	971
	Endosperm	200	301	359	300	179	183	326	241	129	175	413
Tyrosine	Embryo**	1	†	194	414	671	1,316	919	263	363	350	92
	Endosperm	167	†	209	235	292	201	420	9	84	167	495
Valine	Embryo**	1	10	556	838	1,330	1,616	1,655	826	1,325	1,949	2,700
	Endosperm	167	532	392	354	407	325	346	257	276	316	494

* Differences between greenhouse and 5° C. significant at 5 per cent level.
** Differences between greenhouse and 5° C. significant at 1 per cent level.
† None present or present in amounts too small to be measured by technique used.

the alanine content of the endosperm rises along with that of the embryo, production of this compound in the endosperm must be taking place at a rapid rate. In the greenhouse, on the other hand, the embryo apparently uses alanine at the expense of the original supply in the endosperm. This is indicated by a gradual increase in the amount in the embryos in the greenhouse and the decreasing amount in the endosperm portion of the seed at this temperature. Endosperm tissue in dry or moist, ungerminated seeds contained more alanine than that in germinated seeds, regardless of the stage of germination.

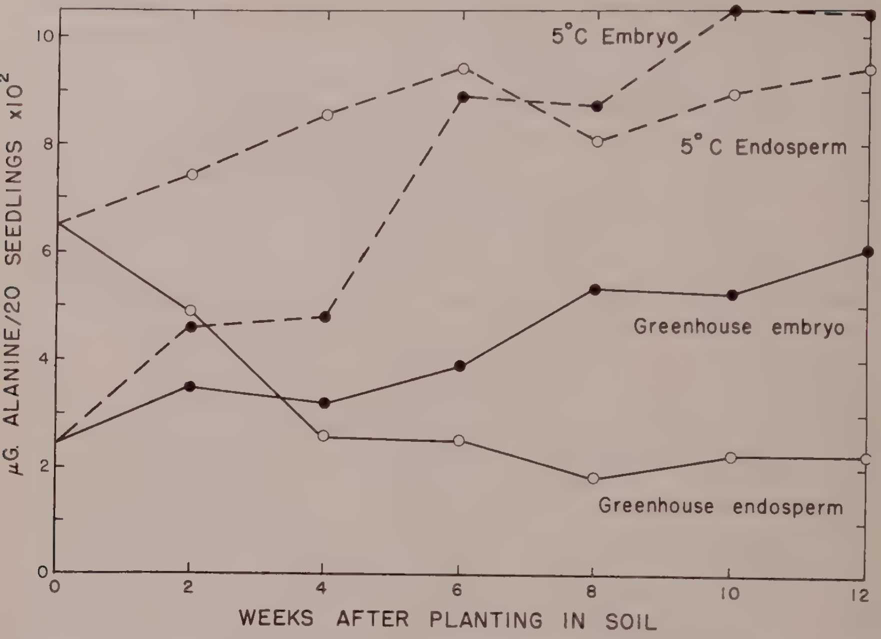

FIGURE 1. Alanine content of embryo and endosperm tissues of germinated seeds of tree peony planted in soil and held in the greenhouse or at 5° C. for 2 to 12 weeks. Averages of four replicate determinations.

Glutamic acid. The rate of glutamic acid accumulation in the embryo at 5° C. is significantly greater than that at greenhouse temperatures (Table I). In both cases, however, the actual amounts increase with time while those of the endosperm are variable. Endosperm of dry and of moist ungerminated seeds is high in glutamic acid content. It appears, then, that there is no synthesis of glutamic acid at either temperature, but the supply is used more rapidly at 5° C.

Glutamine. Although this amide is practically absent from the embryos and is present in very small quantities in the endosperm of dry or moist ungerminated seeds, it is produced rapidly upon germination and thereafter is present in large amounts, especially in the growing embryos (Table I). Twelve weeks after planting, 20 embryos grown in the greenhouse contained 13,488 μg. of glutamine, but 20 embryos held in soil at 5° C. contained 42,850 μg., or more than three times the amount found in greenhouse embryos. The endosperm at 5° C. also contained more glutamine than corresponding tissue in the greenhouse.

Glycine. Because of the relatively small amounts of this acid and the variability of the results from one testing period to another, differences in behavior of glycine between greenhouse temperatures and 5° C. could not be established with certainty. However, it appears that the endosperm accumulates larger amounts of this acid at 5° C. than in the greenhouse (Table I).

Serine. Larger quantities were found in the endosperm at 5° C. than in the same tissue in the greenhouse. There was also a trend in this direction in the embryos though this could not be demonstrated statistically due to the wide scattering of replicate values.

Valine. The increased rate of formation of valine was greater in the embryos at 5° C. than in the greenhouse (Table I). While this difference was highly significant, the quantities of valine in the endosperm remained the same at the two temperatures. This indicates that this amino acid was being produced in the endosperm tissue at both temperatures to provide the embryos with their requirements, but more rapid formation must have been taking place in the endosperm at 5° C. since more was being transported to the embryo there and the endosperm supply was not depleted.

AMINO ACIDS WHICH ACCUMULATE AT GREENHOUSE TEMPERATURE

Asparagine. Although temperature does not seem to affect the aspartic acid content in either embryos or endosperm, the corresponding amide increases much more rapidly and in greater quantity in the greenhouse (Table I and Fig. 2). The possible relationship between the large amounts of asparagine in the growing seedlings in the greenhouse as opposed to the large accumulation of glutamine in seedlings during after-ripening at 5° C. will be discussed below.

Phenylalanine. This acid accumulated rapidly in both embryo and endosperm at 5° C. and in the greenhouse (Table I). No significant differences were demonstrated in the endosperm at the two temperatures during a period of 12 weeks; but the phenylalanine content remained at a higher level in greenhouse seedlings than in those at 5° C. after 10 and 12 weeks.

Tyrosine. Tyrosine content of the embryos in the greenhouse increased rapidly while that of the embryos at 5° C. increased less rapidly up to 6 weeks after which it declined markedly (Table I). Amounts of this acid increased in the endosperm at both temperatures during the 12 weeks of growth in the greenhouse or after-ripening at 5° C.

AMINO ACIDS NOT AFFECTED BY TEMPERATURE

Amino acids which did not appear to depend upon temperature treatment of the seedlings for accumulation were γ-aminobutyric acid, aspartic acid, leucine, lysine, proline, and threonine. This is not to say that the quantity present in either embryo or endosperm or both was not affected by the length of time they had been planted. γ-Aminobutyric acid increased 10-fold in the embryo during the 12 weeks in soil, while the con-

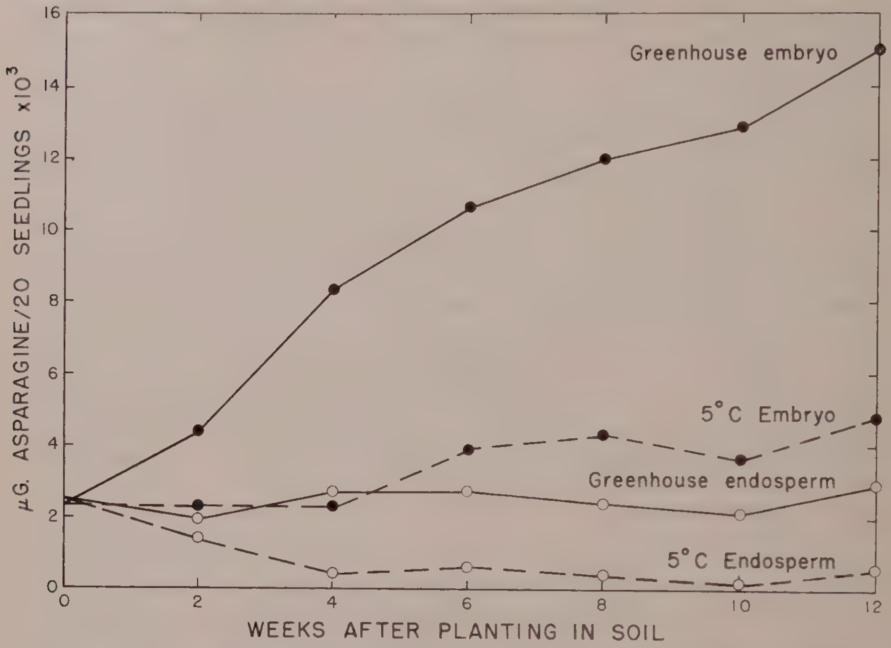

FIGURE 2. Asparagine content of embryo and endosperm tissues of germinated seeds of tree peony planted in soil and held in the greenhouse or at 5° C. for 2 to 12 weeks. Averages of four replicate determinations.

tent of this acid in the endosperm remained constant. Leucine and threonine followed the same pattern as γ-aminobutyric acid, though the embryo contents in these cases were only doubled or tripled. The increase in lysine in the embryos from zero at the time of planting to over 1000 µg. 12 weeks later is striking. Proline also increased in the embryos with time, though results here were very erratic.

Aspartic acid showed at least a three-fold increase in the embryo 12 weeks after planting, with a corresponding decrease in endosperm content of this acid.

ARGININE AND UNIDENTIFIED SUBSTANCES

Arginine and some unidentified substance or substances were present in large quantity on all chromatograms, but, since they were not separated from each other in the procedure used, it remains for future experiments to assess the significance of the arginine and identify the one or more unknowns. From visual observation, it appears that there were larger quantities of these combined substances in seedlings grown in the greenhouse than in those held at 5° C.

The ninhydrin-reactive spot combined with arginine was stable to 1*N*

HCl hydrolysis and produced no color when treated with Sakaguchi, isatin or platinic iodide reagents. Its Rf value was not altered by omitting ammonia from the phenol chamber.

THE EFFECT OF GIBBERELLIC ACID ON AMINO ACID CONTENT

Since gibberellic acid has been found to promote the growth of dormant epicotyl of tree peony, replacing the need for low temperature pretreatment for the production of green shoots in the greenhouse (1), assays of amino acid contents of treated germinated seeds were made. Plantings were made in soil in the greenhouse immediately after treatment of each seedling with 100 μg. of gibberellic acid. Since seedlings so treated emerged above the soil, forming a green shoot, within 2 to 3 weeks after treatment, extracts for free amino acids were made of embryos and endosperm tissue 0, 2, 4, 7, 9, 11, 14 and 22 days after treatment and planting.

The results are shown in Table II, where the figures represent averages of four replicate determinations (extracts of two samples of seedlings with duplicate analyses run on each extract) for each sampling.

A strict comparison of gibberellic acid-treated seedlings with untreated ones at either greenhouse temperature or 5° C. is not possible because of the difference in time involved. For example, only 2 to 3 weeks are required for gibberellic acid to bring about green shoot production in the greenhouse without any low temperature pretreatment. On the other hand, 12 weeks at 5° C. are required to prepare the epicotyl for green shoot production upon transfer to the greenhouse. In spite of this difference certain trends may be noted. When the amino acid contents 2 or 3 weeks after treatment with gibberellic acid were compared with those in untreated embryo or endosperm after 2 or 4 weeks in the greenhouse or at 5° C., it was seen that, with the exception of alanine, the trends resembled those in the greenhouse without gibberellic acid treatment rather than those which are after-ripened at 5° C. Comparison of final measurements made when the after-ripening was complete, i.e. after 3 weeks' growth of gibberellic acid-treated seeds in the greenhouse or 12 weeks' after-ripening at 5° C., indicated similar relationships (Tables I and II).

THE EFFECT OF EMBRYO AND ENDOSPERM FRESH WEIGHTS ON AMINO ACID CONTENT

Twenty embryos of dry seeds had an average fresh weight of about 13 mg. The weight of the same number of moist, ungerminated embryos was about 58 mg. This increased to more than 1000 mg. by the time the seed germinated to form roots. Fresh weights of endosperm tissue from 20 dry or moist, ungerminated seeds were approximately 3545 and 5560 mg., respectively.

Fresh weights of tissues from germinated seeds should be indicative of

TABLE II

AMINO ACID CONTENTS OF EMBRYO AND ENDOSPERM TISSUES OF GERMINATED SEEDS OF TREE PEONY TREATED WITH 100 μG. EACH OF GIBBERELLIC ACID AND HELD IN SOIL IN THE GREENHOUSE FOR 2 TO 22 DAYS

Amino acid	Extract	μG./20 germinated seed days after gibberellic acid treatment							
		0	2	4	7	9	11	14	22
Alanine	Embryo	243	241	264	280	319	392	427	459
	Endosperm	783	809	567	587	776	430	854	600
γ-Aminobutyric acid	Embryo	87	131	137	201	187	217	167	483
	Endosperm	531	325	267	367	376	101	166	152
Asparagine	Embryo	728	1,436	1,801	2,216	3,288	4,000	4,892	8,251
	Endosperm	1,117	999	734	1,421	1,368	1,125	1,702	1,467
Aspartic acid	Embryo	166	275	217	492	363	650	529	750
	Endosperm	1,616	1,426	833	1,379	984	841	1,366	775
Glutamic acid	Embryo	361	475	675	917	525	1,151	872	1,516
	Endosperm	4,426	5,433	3,093	6,079	3,566	3,575	3,233	3,602
Glutamine	Embryo	3,543	4,894	6,063	9,122	8,250	13,083	10,997	13,500
	Endosperm	1,668	1,251	1,116	1,661	1,674	1,051	1,317	925
Glycine	Embryo	0	0	0	0	0	0	0	0
	Endosperm	109	25	42	75	100	0	46	88
Leucine	Embryo	485	369	457	451	588	802	604	750
	Endosperm	317	200	150	190	241	207	283	193
Lysine	Embryo	99	125	193	308	237	375	500	816
	Endosperm	433	258	182	208	326	84	142	67
Phenylalanine	Embryo	265	256	254	217	325	192	150	450
	Endosperm	834	541	583	525	759	383	500	409
Proline	Embryo	14	47	73	84	50	150	13	150
	Endosperm	261	133	87	150	167	17	50	108
Serine	Embryo	255	288	350	400	410	600	434	701
	Endosperm	635	525	423	599	600	352	517	493
Threonine	Embryo	224	263	314	367	425	556	467	617
	Endosperm	484	351	268	347	342	135	234	160
Tyrosine	Embryo	355	288	361	342	462	509	500	517
	Endosperm	467	226	209	225	293	133	141	59
Valine	Embryo	547	556	612	635	748	1,067	992	367
	Endosperm	609	291	259	372	388	283	300	275

growth of the embryo and depletion of the food reserves in the endosperm with increased time in soil. Some of these weights are shown in Table III.

A general increase in embryo weight is to be noted up to 12 weeks, either in the greenhouse or at 5° C., with the greater increase at 5° C. especially after 8 weeks or longer in soil. Differences are negligible up to 8 weeks. On the other hand, amino acid differences are evident after 2 weeks in most cases and are marked before 8 weeks (Table I, Figs. 1 and 2) when the first weight differences become apparent. Similarly, endosperm amino acids vary greatly in the greenhouse and at 5° C. in spite of the slight variation in actual fresh weights of these portions (Tables I and III). Obviously, then, the increased or decreased quantities of free amino acids reported above do not depend upon fresh weights, either of embryo or endosperm.

TABLE III

FRESH WEIGHTS OF EMBRYO AND ENDOSPERM TISSUES OF GERMINATED SEEDS OF TREE PEONY PLANTED IN SOIL AND HELD IN THE GREENHOUSE OR AT 5° C. FOR 0 TO 12 WEEKS

Weeks after planting	Fresh weights* (mg.) after period in greenhouse or at 5° C.			
	Embryo		Endosperm	
	Greenhouse	5° C.	Greenhouse	5° C.
0	1026	1026	4966	4966
2	1696	1278	4432	4356
4	2197	2133	4331	4812
6	2233	2843	4733	4795
8	2807	3597	4853	4230
10	3091	3824	3735	4242
12	3344	5301	3935	4311

* Average of two lots of 20 each.

It should be pointed out that embryos increase in weight at 5° C. over that attained in the greenhouse after 8 to 12 weeks in soil in spite of the more extended growth of the root system at the higher temperature. The increased weight at 5° C. can be attributed, at least in part, to a much thickened epicotyledonary axis (1). Embryo weights of seedlings treated with gibberellic acid and planted in soil in the greenhouse increased up to 2065 mg. after 11 days as compared with 1696 for greenhouse controls in 2 weeks. This increased weight may also be due to a thickened epicotyledonary axis, which resembles that produced after a longer time interval at 5° C. (1).

It is not known at present whether a correlation exists between dry weights and free amino acid contents of embryo or endosperm tissues.

EVALUATION OF THE METHOD

In Table IV values for alanine content are shown as samples of the variation in four replicate determinations. The standard deviation of the average of the four determinations, consisting of duplicate analyses on each of two extracts, expressed as percentage of the average, gave values of 9.2 and 15.9 for embryos from the greenhouse and 5° C. respectively, and 10.6 and 17.6 for endosperm under the same conditions.

The use of duplicates of two extracts involving 20 seedlings each, separated into embryo and endosperm components, permits an analysis to determine whether most of the error is due to sampling or to the procedure used. The standard deviation of the average of the four replicates, consisting of duplicate analyses made on two extractions, was calculated from the formula:

$$S_{av} = \sqrt{\frac{S_e^2 + S_a^2/2}{2}}$$

TABLE IV

ALANINE CONTENT OF EMBRYO AND ENDOSPERM TISSUES OF GERMINATED SEEDS OF TREE PEONY PLANTED IN SOIL AND HELD IN THE GREENHOUSE OR AT 5° C. FOR 2 TO 12 WEEKS. FOUR REPLICATE DETERMINATIONS CONSISTING OF DUPLICATE ANALYSES OF EACH OF TWO EXTRACTS

Tissue	Weeks after planting	μG./20 germinated seeds							
		Greenhouse				5° C.			
		Extract 1		Extract 2		Extract 1		Extract 2	
		a	b	a	b	a	b	a	b
Embryo	0	263	238	242	225	263	238	242	225
	2	300	375	350	375	600	613	325	325
	4	334	284	326	326	400	400	576	551
	6	400	367	367	425	966	966	801	825
	8	534	666	500	434	1000	999	700	800
	10	500	400	599	599	867	833	1200	1301
	12	600	567	626	626	999	968	1200	1002
Endosperm	0	583	603	684	750	583	603	684	750
	2	516	483	501	450	667	917	675	701
	4	216	317	251	251	385	884	1100	1049
	6	251	251	251	267	674	674	1175	1251
	8	150	133	249	201	766	667	901	901
	10	200	200	251	233	783	783	1049	968
	12	284	251	186	167	975	975	934	883

where S_e^2 is the estimated variance for extraction; S_a^2 is the estimated variance for analyses; and S_{av} is the standard deviation of the average of four determinations, duplicates on each of two extractions (20, p. 34).

Combining all treatments and replicates, 63.3 per cent of the variance was found to be due to the extract, or the sample, while 36.7 per cent was due to the duplicates, or the analytical procedure. More than 90 per cent of the error could be attributed to sampling at the time the seedlings were removed from the granulated peat moss and planted in soil. This shows the extreme variability in free amino acid content of seedlings with roots 25 to 35 mm. long, and indicates more careful selection of seedlings should be made for future tests. It has been shown that seedlings with longer roots respond more quickly to gibberellic acid treatment than those with shorter roots (1), the former probably representing a significant advance in development over the latter. Seedlings kept in soil in the greenhouse or at 5° C. for 12 weeks showed a greater percentage (11 to 71) of analytical error, with corresponding decreases in the percentage error due to sampling.

Replicate values were also analyzed according to the amino acid measured. Glutamic acid, glycine, lysine and serine showed 14 per cent or less of analytical error. Between 18 and 30 per cent of the error was due to duplicates in determinations of asparagine, aspartic acid, leucine, threonine and valine. Alanine, γ-aminobutyric acid, glutamine, phenylalanine, and tyrosine duplicates accounted for 45, 64, 43, 79 and 45 per cent, respectively, of the replicate variance. The number of micrograms of a specific amino acid applied in the spots on the chromatograms varied with the change in composition of the seed but could not account for duplicate variance which was greater in these experiments than reported by Porter *et al.* (11). However, the over-all error due to sampling was roughly twice that due to duplication, so the precision of the method is perhaps adequate for this material.

In spite of the variability of the results, significant differences in amino acid content have been demonstrated between seedlings held in the greenhouse and seedlings after-ripened at 5° C. It will be recalled that under greenhouse conditions green shoots do not appear without pretreatment either with cold or with gibberellic acid. With more refined techniques, other differences shown by the present data, such as the downward trend of leucine content in seedlings in the greenhouse while the quantity of this amino acid remained high in those at 5° C., may be proved significant.

DISCUSSION

Three recent review articles have emphasized the importance of nitrogen metabolism in the plant (16, 18, 19). In the latest of these (19), the authors point out that there is no general agreement on whether or not amino acids are precursors in the synthesis of proteins in plants. They believe, however, that studies of the assimilation of inorganic nitrogen by plant cells and of their enzymic properties have strengthened the position of glutamic acid as the chief primary product in the synthesis of amino acids. Further metabolism of the primary products of nitrogen assimilation is generally considered to involve extensive interconversion of amino acids, either by transamination or by interconversion of carbon skeletons, resulting in the numerous amino acids commonly found in plant tissue. The authors further state that results of all experiments given in the review confirm the importance of amides and of arginine as the chief compounds involved in the mobilization and transfer of nitrogen.

Nitrogen metabolism during the after-ripening process in which changes necessary for subsequent germination or growth take place in dormant embryos or parts of embryos has not been elucidated up to the present time. However, some literature is available on biochemical changes involving nitrogenous compounds in the germination of nondormant seeds.

Folkes and Yemm (4) found that the endosperm of barley grain was capable of supplying three-fourths of the total amino acid requirement of the embryo during germination and the amounts strongly suggested that amino acids were being transported to the embryo and metabolized there.

Extensive interconversions of amino acids accompanied the synthesis of new proteins in the embryo at the expense of storage proteins of the endosperm. Among the amino acids these authors found to be increased during germination of barley are aspartic acid, alanine, glycine, lysine and arginine. At the same time there were sharp declines in amounts of glutamic acid, amide and proline. Their tests showed that glutamine occupied a central position in these reactions.

Aspartic and glutamic acids and their amides appeared in large quantities upon germination of wheat, rice or radish seeds according to Kojima *et al.* (7). Nuccorini (9) found that glutamine was present in much smaller quantities than glutamic acid in germinated seeds of wheat and flax, whereas the reverse was true with asparagine. He concluded that glutamine was involved in the metabolism of germinating seed but asparagine was not. The use of C^{14}-labeled glutamic acid during germination of gram seeds indicated rapid catabolism and gave strong evidence for the synthesis of glutamic acid according to Sivaramakrishnan and Sarma (14). Aspartic acid and asparagine were products of glutamic acid degradation according to these authors. Smith and Williams (15) found that glutamic-aspartic transaminase increased more rapidly than glutamic-alanine enzyme during the first 120 hours of germination of seeds of Gramineae, Leguminosae and Cucurbitaceae. Hatano (5, 6) has shown that asparagine appears in coniferous and *Ginkgo biloba* seeds only upon germination.

In all of these cases comparisons were made between the contents of the dry seeds and those of the germinating seed and/or the nondormant seedling at ordinary temperatures for plant growth. In the present experiments, free amino acids were determined in the embryos and endosperm of dry seeds and of moist ungerminated seeds, but the main comparisons were made between seeds already germinated and with the roots growing at greenhouse temperature (where the bud which forms the green shoot is dormant) and at 5° C. (where the growth of the roots is slowed down, but where the dormancy of the epicotyl is broken so that a green shoot is produced upon transfer to greenhouse temperature). In a sense, then, this represents a straight growth phenomenon in the extension of the nondormant root, and an after-ripening phenomenon in the case of the dormant epicotyl.

Differences in free amino acid contents under these two conditions suggest that certain acids are related to the after-ripening as opposed to the straight growth process. Increased amounts of glutamine and decreased amounts of asparagine in both embryo and endosperm at 5° C. as compared with those in the greenhouse, indicate that glutamine may have an important role in after-ripening, while asparagine accumulates in embryos with growing roots, but with dormant epicotyls. Meiss (8) found that asparagine accumulation proceeded with growth of the hypocotyl of etiolated seedlings of *Lupinus albus* L. while epicotyl growth was inversely related to these two processes. It should be pointed out that tissue differentiation in the epicotyledonary axis of the tree peony embryo takes place at both greenhouse temperature and 5° C., but this differentation is followed by elongation to form a green shoot above ground if the seedling has been exposed to the low temperature, while this does not happen if untreated seedlings are kept in the greenhouse (1). Hence it appears that accumulation of glutamine is part of the biochemical requirement for after-ripening, and is not directly related to morphological differentiation.

Rijven (13) points out that recent biochemical literature ascribes an important primary role in plant nitrogen metabolism to glutamine and glutamic acid, and considers the accumulation of asparagine the result of catabolic processes. In the light of these findings, it appears that development of the nondormant root in the greenhouse may be made possible by the proteolysis of the endosperm proteins, while at 5° C. synthesis as well as proteolysis is taking place. Rijven (13) also found that growth of 12 species of plant embryos *in vitro* was superior when supplied with glutamine, but was inhibited by asparagine at concentrations of more than 10 mg./l. Large concentrations of asparagine were more inhibitory in the absence of glutamine. He concluded that a large surplus of external asparagine may block the main glutamine pathway, thus bringing about a depression of growth. Whether large quantities of endogenous asparagine accumulated in tree peony embryos in the greenhouse could block after-ripening of the embryo at this temperature is a question but its presence is suggestive.

The relationships between the quantities of aspartic and glutamic acids and their corresponding amides as well as the relationship of the two amides to each other have been studied using the data obtained in the present tests.

In the endosperm of the dry seed the ratio of asparagine to aspartic acid is 8 to 10. In the moist ungerminated seed there is only half as much asparagine as aspartic acid. Upon germination the endosperm contains slightly more asparagine than aspartic acid and in the embryo after germination there is five times as much asparagine as aspartic acid. The proportion of asparagine to aspartic acid continues to increase with growth of the root in the greenhouse so that after 12 weeks there is ten times as much amide as acid in the embryo and about four times as much in the endosperm. At 5° C., the situation is different. Here the amount of asparagine in relation to aspartic acid has actually decreased after 12 weeks over that present at the beginning of the cold treatment. Also, actual amounts of both of these substances are small at 5° C. as compared with the greenhouse temperature. This may indicate the importance of these metabolites in growth of root and differentiation of tissues of the epicotyl in the greenhouse, but their production is apparently not essential to the after-ripening process. Asparagine may be interconverted to other acids under conditions which bring about after-ripening.

The ratio of glutamine to glutamic acid in the endosperm of dry seeds shows very small amounts of the former (1 to 20). In moist ungerminated seed, the ratio is even lower (1 to 300). After germination the ratio goes up to 1 to 5 in the endosperm and 9 to 1 in the embryo. This high proportion of glutamine is maintained in the greenhouse but even more glutamine is mobilized in the embryo at 5° C. (14 to 1). In the endosperm the ratio of glutamine to glutamic acid remains the same at the two temperatures though the relative quantities are higher at 5° C. Production of glutamic acid by hydrolysis of endosperm protein and especially its conversion to glutamine, appears to be related to the after-ripening process at 5° C.

An examination of the glutamine-asparagine ratio in tree peony shows a low proportion of the former in the endosperm of dry seeds and an even lower proportion in endosperm of moist, ungerminated seeds. With germination to form a root the ratio goes up with an increase in quantity. This level is maintained in the greenhouse but rises sharply at 5° C. so that the endosperm at the low temperature has three times as much glutamine as asparagine. There is an increase in the ratio of glutamine to asparagine in the embryo at germination, followed by a slight decrease in the greenhouse and a four-fold increase at 5° C. Steward and Pollard (16) found that when tissues of potato and carrot were made to grow by the addition of the growth factors in coconut milk, the balance between asparagine and glutamine changed in favor of the glutamine, the asparagine disppearing. Asparagine does not disappear in tree peony seedlings held at 5° C. for after-ripening, but it is reduced to about one-third the quantity present in seedlings at greenhouse temperature while the glutamine content increases to three times the quantity present in greenhouse seedlings. These changes accompany the extension of the root system and differentiation of tissues of the shoot axis in the greenhouse, but no extension of epicotyl length as occurs at 5° C. (1).

Steward *et al.* (17) point out that the three closely related amino compounds, glutamine, glutamic acid, and γ-aminobutyric acid, are present in greater quantity in the meristem of *Lupinus albus* than elsewhere in the plant; and that other amino acids, especially the amide, asparagine, are more concentrated in the older leaves. They state (17, p. 133), "The relationships of aspartic acid and asparagine, on the one hand, and glutamic acid and glutamine, on the other, to development all suggest that the latter compounds are the more nearly related to metabolic activity and to protein synthesis in the meristem itself, while asparagine is formed mainly in regions in which growth is slowing down or has ceased."

According to Webster (18), aspartic and glutamic acids and alanine occupy unique positions in the metabolism of the cell and may act as precursors of other amino acids. It will be recalled from the data presented above that alanine is also more abundant in tree peony seedlings with after-ripened epicotyls (at 5° C. or in the greenhouse after gibberellic acid treatment) than in those which remain dormant at greenhouse temperature. The role of alanine as well as that of valine which tends to accumulate in the tree peony embryo at 5° C. is still a question. Also the reason for the increased quantities of glycine and serine in the endosperm at 5° C. and of phenylalanine and tyrosine in the embryos held in the greenhouse must await further tests. Arginine and unidentified acids may well be important in the nitrogen metabolism of tree peony seedlings, but procedures employed in the present experiments did not permit their isolation and measurement.

The after-ripening of the dormant epicotyl of the tree peony seedling apparently depends upon biochemical changes within the tissues, since morphological differentiation takes place in the dormant epicotyl (1). At least some of the necessary chemical changes may involve a synthesis of proteins in the region of the dormant epicotyl which, in turn, would depend upon a movement of amino acids to the region from the endosperm where they are stored in the form of free amino acids or proteins. Such an explanation has been suggested by Cotrufo and Levitt (2) to explain the sprouting of potato tubers. Thus both growth and after-ripening processes result from a proteolysis of protein and a transfer of amino acids to the embryo with a subsequent synthesis to prepare for germination (to after-ripen) or contribute to the growing embryo. Further tests to obtain more exact information on nitrogen metabolism, including total nitrogen and protein studies, are contemplated. Other compounds, such as keto acids, nucleic acids and carbohydrates (19), are known to be involved in the growth processes. Their roles in the after-ripening process are still to be determined.

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SYNERGISM OF THE CARBAMATE INSECTICIDES

HERBERT H. MOOREFIELD[1]

SUMMARY

The activity of the carbamate insecticides can be synergized with the pyrethrins adjuvants. Tests of topical application on house flies demonstrated that both aromatic and heterocyclic carbamates can be potentiated with each of the following methylenedioxyphenyl synergists: piperonyl butoxide, Sesoxane, Sulfoxide, *n*-propyl isome and sesamine oil extractives. This phenomenon was also evident in fly bait tests, with bean aphid sprays and by topical application to the German cockroach.

Isolan and Pyrolan were synergized about 10-fold and this was reflected in transposition of the house fly dosage-mortality line. The dosage mortality response of the house fly treated with Sevin (1-naphthyl *N*-methylcarbamate, or 4-*tert.*-butylphenyl *N*-methylcarbamate was a broken line. When these compounds were combined with a synergist, the curve was straightened, the slope of the curve steepened, and the whole translated. Such mixtures make possible control of the house fly with carbamates that are otherwise ineffective fly toxicants.

INTRODUCTION

The synergism concept, wherein the activity of combinations of compounds elicit biological responses greater than the additive effects of individual components, is well known and widely practiced in the field of insect control. Mixtures of most of the proprietary insecticides have been evaluated, and several specific chemicals have been produced for the express purpose of enhancing the insecticidal properties of various toxicants. Scattered results of these researches have been brought together in recent years, and our present knowledge of the synergism phenomenon has been critically reviewed (8, 11, 12).

With the appearance of a novel class of insecticidal compounds, the carbamic acid esters (4), and their subsequent exploration (5, 7), the possibility of incorporating the many advantages of synergism should be appraised. It was the objective of the present studies to determine if the carbamates could be synergized and to what extent the toxicity might be potentiated with relatively harmless adjuvants.

MATERIALS AND METHODS

The house flies (*Musca domestica* L.) used in these experiments were all four-day-old females of the NAIDM strain. Compounds were applied to the dorsal thorax in 1 μl. volumes of acetone by means of a micrometer-driven syringe while the flies were held under CO_2 anesthesia. Treated flies were supplied sugar water for nourishment and mortality counts were made after 18 hours. The data reported are the average results obtained from four replicates of 25 flies each. Bait feeding tests were conducted with the compounds dissolved in a minimum of acetone and diluted in an emulsified 10 per cent sugar solution. Fifteen ml. of this mixture were added to a small paper cup containing a one-inch square of absorbent "Kempack." Twenty-five flies were restrained with the bait under a five-inch hemispherical screen cage and the dead flies counted after 24 hours.

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Evaluations with the German cockroach (*Blattella germanica* L.) were performed on adult males by topical application. Compounds contained in 1 μl. doses of acetone were delivered on the thoracic dorsum with the micrometer-syringe and the dead insects were counted 24 hours later. Each test was performed in quadruplicate.

Spray tests were carried out with the bean aphid (*Aphis fabae* Scop.). Potted *Nasturtium* sp. plants, infested with aphids, were trimmed to contain approximately 100 insects per plant. The pots were placed on a revolving turntable and the compounds (formulated in acetone, emulsifier and water) were applied to run-off with a DeVilbiss spray gun set to deliver 100 ml. in 30 seconds. The treated plants were placed horizontally over a piece of white, ruled paper, and after 24 hours the dead aphids were counted. The reported results are an average of four replicated tests.

RESULTS

Insecticidal carbamates of different structure and a wide selection of the currently used methylenedioxyphenyl synergists were chosen for this study. The 3-*tert.* butylphenyl *N*-methylcarbamate, reported to be the most effective substituted phenyl carbamate (7) and Sevin (1-naphthyl *N*-methylcarbamate), a bicyclic, were selected as aromatic representatives. Two heterocyclics were also included, Pyrolan (1-phenyl-3-methylpyrazolyl-5 dimethylcarbamate), with an aryl moiety and Isolan (1-isopropyl-3-methylpyrazolyl-5 dimethylcarbamate), with an alkyl substituent. Table I shows that mixtures of any of these carbamates with one of the following synergists, delivered topically onto adult house flies, will demonstrate definite synergism: piperonyl butoxide (3,4-methylenedioxy-6-propylbenzyl *n*-butyl diethylene glycol ether), Sesoxane [2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane], Sulfoxide (*n*-octyl sulfoxide of isosafrole) *n*-propyl isome (di-*n*-propyl 6,7-methylenedioxy-3-methyl-1,2,3,4-tetrahydronaphthalene-1,2-dicarboxylate) or sesamine oil extractives (John Powell & Co.). Extremely high levels of potentiation were obtained with selected concentrations of the compounds; for example, Sevin at 0.2 μg. per fly and piperonyl butoxide at 2.0 μg. are each completely nontoxic to the house fly, but applied in combination result in 100 per cent mortality. The highest degrees of carbamate synergism were obtained with Sesoxane, piperonyl butoxide, and Sulfoxide. The *n*-propyl isome was intermediate

TABLE I

EFFECTIVENESS OF SEVERAL CARBAMATE-SYNERGIST COMBINATIONS AGAINST THE HOUSE FLY WITH TOPICAL APPLICATIONS

Carbamate	Dose* (μg.)	Percentage mortality in combination with synergist									
		Piperonyl butoxide		Sesoxane		Sulfoxide		*n*-Propyl isome		Sesamine oil extractives	
		1.25	2.5	1.25	2.5	1.25	2.5	1.25	2.5	1.25	2.5
3-*tert*. Butylphenyl *N*-methylcarbamate	0.25	100	—	100	—	98	—	74	—	29	—
	0.5	—	100	—	100	—	100	—	100	—	49
Sevin	0.25	92	—	100	—	48	—	15	—	9	—
	0.5	—	100	—	100	—	93	—	76	—	65
Isolan	0.25	100	—	100	—	100	—	96	—	76	—
	0.5	—	100	—	100	—	100	—	100	—	89
Pyrolan	0.25	100	—	100	—	57	—	100	—	35	—
	0.5	—	100	—	100	—	92	—	100	—	40

* All carbamates and synergists applied singly at doses indicated produced less than 10 per cent mortality.

in effectiveness, and sesamine oil extractives (the concentration corrected for 100 per cent active ingredients) gave a comparatively poor performance.

The carbamate synergism was examined in terms of effect on the house fly dosage-mortality response. In ratios of synergist to carbamate ranging from equal parts to 20:1, potentiation was evident, with the maximum response occurring in mixtures of 5 to 10:1. The increased activity obtained with mixed doses was apparent over the entire scope of the dosage-mortality relationship and was reflected by each of the carbamates studied (Fig. 1).

Sevin and 3-*tert*. butylphenyl *N*-methylcarbamate are both inefficient fly toxicants as shown by their dosage-mortality curves which break at about 70 to 80 per cent mortality. Used alone, a large dose is required to reflect a small increase in toxicity. Complete fly control, exemplified by 100 per cent kill, is virtually impossible to attain with these chemicals as 40 μg. per fly is little more effective than a dose of 5 μg. Fortification of these carbamates with piperonyl butoxide resulted in a straight dosage-mortality line and displaced it to the left indicating an increased efficiency of kill. Further, the slope of the line was steepened showing a more homogeneous population response. Where a dose of 40 μg. per fly of Sevin applied alone effected 70 per cent mortality, equivalent results were obtained with only 0.12 μg. of Sevin in combination with 0.6 μg. of piperonyl butoxide.

Synergism of the carbamates can also be shown with other insect species and by various methods of application. Table II presents data showing Sevin synergized by piperonyl butoxide against the house fly in bait tests, the bean aphid with sprays, and the German cockroach by topical application of microdrops. In the fly bait tests the kill was rapid, probably indica-

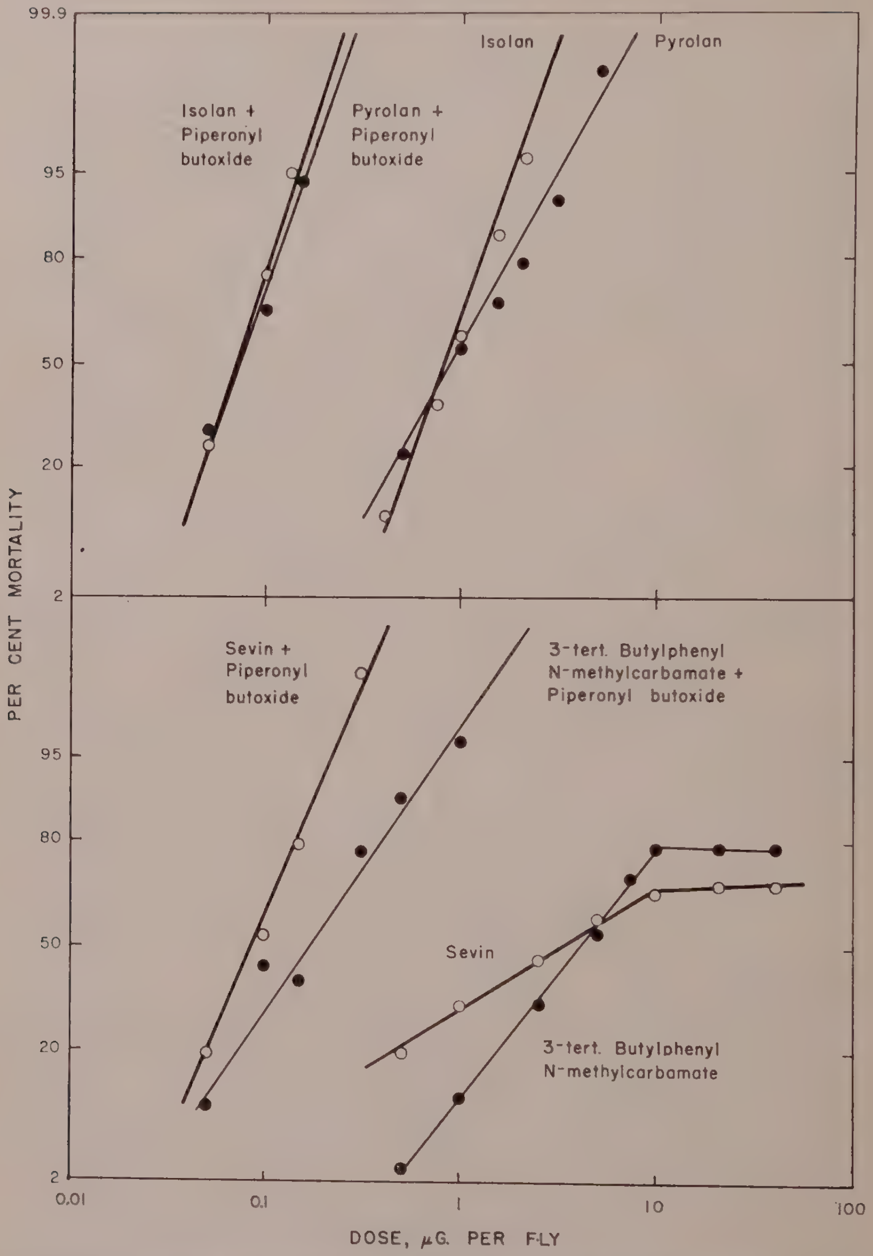

FIGURE 1. Effect of synergizing carbamate insecticides on dosage-mortality response of the house fly. Ratio of carbamate to piperonyl butoxide, 1:5. Piperonyl butoxide applied alone effected no mortality at the doses used.

TABLE II

CARBAMATE SYNERGISM WITH DIFFERENT INSECTS AND WITH COMPOUNDS VARIOUSLY APPLIED

Dose*		Percentage mortality of insects		
Sevin	Piperonyl butoxide	Bean aphid (spray)	House fly (bait)	Cockroach (topical)
0.2		0		
2		40		
5			3	0
10			28	5
	2	0		
	20	0		
	50		0	0
	100		0	0
0.2	2	25		
2	20	94		
5	25		60	
10	50		100	
5	50			90
10	100			100

* Dosage of bean aphid sprays and house fly baits expressed in mg./100 ml. Topical applications expressed as μg./roach.

tive of contact activity rather than stomach action. Dosage-mortality results obtained by bait feeding were similar to those based on topical application.

DISCUSSION

The fact that the cholinesterase-inhibiting insecticides can be synergized is not a new finding. Investigations on the use of synergists with organic phosphates against the body louse were first reported in 1954 (2), and similar results were found when residues of pyrethrins synergists combined with certain phosphates were tested against DDT-resistant house flies (6). Further research on the potentiation of the phosphates with piperonyl butoxide revealed that although the phenomenon was demonstrable with several different insecticides of this class (10), such results were not predictable. Definite antagonism has been described for mixtures of piperonyl butoxide and malathion against strains of both DDT-susceptible and resistant house flies (9).

Recent studies have pointed out that the joint action of piperonyl butoxide with anticholinesterases such as parathion are synergistic when applied to the crustacean, *Daphnia magna* (3). In like manner, increased diaphniacidal activity can be obtained with piperonyl butoxide and either natural (eserine) or synthetic (Pyrolan) carbamates. The present experiments demonstrate that the insecticidal activity of the carbamates can be augmented with several of the pyrethrins synergists and that this synergism can be shown with unrelated species of insects. That carbamates of diverse chemical structure are all about equally potentiated can be interpreted to mean that this class of insecticides will react more uniformly to being synergized than have the organic phosphates.

The broad activity that the methylenedioxyphenyl synergists have displayed in combination with insecticides of radically different chemical structure (pyrethrins, phosphates, carbamates, rotenone, chlorinated hydrocarbons) suggests that the current concept of these synergists acting as inhibitors of detoxication mechanisms (8, p. 94) should be more critically assessed. This group of compounds must either possess a polyvalent and nonspecific potential for blocking enzyme catalysis, or act independently at a less critical site in the insect, or influence the efficiency of an essential property common to the reactivity of all successful insecticides such as aiding transport. Elucidation of the mode of action of the synergists should be more productive in studies involving insecticides which serve as anticholinesterases and the toxic action of which is better documented than with compounds such as pyrethrins whose ultimate function is still somewhat obscure.

The significance of the discontinuous dosage-mortality response effected by single application of the aromatic carbamates tested in this work is not understood. This characteristic has been previously described and more fully discussed by Bliss (1). Incorporation of a synergist with these compounds straightens the dosage-mortality line, steepens the slope and transposes the whole to the left (Fig. 1). The heterocyclic carbamates, Isolan and Pyrolan, elicit smooth, unbroken, dosage-mortality responses with the house fly. Potentiation of these compounds is manifested by a tenfold increase in toxicity and a corresponding transposition of the curve.

Synergism of Sevin and of 3-*tert.* butylphenyl *N*-methylcarbamate would provide a means of practical house fly control with insecticides that are otherwise unsatisfactory against this pest. The use of synergistic carbamate combinations could provide further advantages such as effectiveness against a wider range of insects, presentation of a greater margin of mammalian safety by mixing a harmless adjuvant with a more toxic carbamate, reduction in cost, and possible suppression of insecticide resistance through the application of mixed toxicants.

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Note

A SYSTEM FOR SEPARATING SULFUR AND NONSULFUR AMINO COMPOUNDS BY TWO-DIMENSIONAL PAPER CHROMATOGRAPHY[1]

David Margolis and Richard H. Mandl

Smith and Tuller (5) have described a useful solvent system for the separation of those organic sulfur compounds normally encountered in animals. Cotty *et al.* (3) have modified this method in order to achieve separation of the sulfur compounds present in an alcoholic extract of insects. However, neither of these techniques, when applied to plant extracts, was found to be as satisfactory in this laboratory as the system described below. This report presents data obtained by separating 25 sulfur compounds, 12 maleimide adjuncts of sulfhydryl compounds, and 39 nonsulfur-containing amino acids, amides and amines with a two-dimensional, descending paper chromatography system.

The sample is applied in aliquots of 5 μl. or less to Whatman No. 3 filter paper. Brom cresol purple, which has a mobility approximately 10 per cent faster than isoleucine in both solvents, is applied as a marker at the base line (4). The purpose of this marker is to serve as a visible guide for the control of the time required for a chromatographic separation.

The chromatogram is developed in the first descending direction with ethanol—*tert.*-butyl alcohol—58 per cent (w/v) ammonium hydroxide–water (60:20:5:15 v/v) (7). The second solvent is *tert.*-butyl alcohol—88 per cent (w/v) formic acid–water (14:3:3 v/v) (1).

If it is desired to determine the presence of compounds reacting with ninhydrin quantitatively, the ammonia is removed by dipping the paper in 1 per cent (w/v) alcoholic potassium hydroxide after the removal of the first solvent. The potassium hydroxide is then neutralized by dipping the paper in 1 per cent (v/v) alcoholic acetic acid. The excess acetic acid is removed by drying in air.

Three reagents are used for locating the compounds on paper. They may be used individually or in succession as listed.

a) *Platinic iodide* (2, p. 131). The reaction is more sensitive if the concentration of the reagents in acetone is doubled.

b) *N-Ethylmaleimide* (2, p. 132). The paper is dipped into 0.05M *N*-ethylmaleimide, dried for 15 minutes and then dipped into 1 per cent (w/v) alcoholic potassium hydroxide. *N*-Ethylmaleimide-thiol derivatives appear immediately as red or pink spots.

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TABLE I

MIGRATION CHARACTERISTICS OF SULFUR AND NONSULFUR COMPOUNDS

Compound	No.	R_{proline}	
		Solvent 1 Ethanol–*tert.*-butyl alcohol–ammonia–water	Solvent 2 *tert.*-Butyl alcohol–formic acid–water
A. Sulfur containing:			
Methionine	50	1.09	1.12
Methionine sulfoxide*	51	1.09	0.64
Methionine sulfoxide	52	0.78	0.64
Methionine sulfone	53	0.71	0.46
Methionine sulfoximine	54	0.52	0.37
Methylmethionine sulfonium chloride	55	0.41	0.38
Ethionine	56	1.32	1.30
S-Methylcysteine	57	1.05	0.94
S-Ethylcysteine	58	1.27	1.12
Cystine	59	0.22	0.13
Homocystine	60	0.29	0.43
Cystine disulfoxide	61	0.27	0.40
Cystamine	62	1.53	0.40
Cystamine disulfoxide	63	0.84	0.54
Cysteinesulfinic acid	64	0.30	0.40
Cysteic acid	65	0.33	0.21
Hypotaurine	66	0.84	0.54
Taurine	67	0.93	0.35
Cystathionine	68	0.19	0.22
Lanthionine	69	0.13	0.13
Djenkolic acid	70	0.17	0.18
Glutathione (oxid)	71	0.01	0.09
N-Acetylglutathione	72	0.04	0.17
Glutamylcystine	73	0.02	0.17
Cystinylglycine	74	0.16	0.17
B. *N*-Ethylmaleimide derivatives:			
Cysteine	75	1.12	1.30
Homocysteine	76	1.11	1.13
Glutathione	77	0.34	0.79
Glutamylcysteine	78	0.36	0.91
Cysteamine	79	1.67	0.84
Thiolhistidine	80	1.74	1.20
Ergothioneine	81	0.60	0.94
Hydrogen sulfide	82	2.08	1.69
Thiomalic acid	83	0.60	0.63
β-Mercaptopyruvic acid	84	1.35	1.38
Thiosulfate	85	1.22	0.30
Sulfite	86	1.69	0.86
C. Miscellaneous:			
Inorganic sulfate	87	0.10	0–0.1 (Streaks)
D. Not containing sulfur:			
Aspartic acid	2	0.22	0.65
Glutamic acid	3	0.29	0.82
Serine	4	0.65	0.64
Glycine	5	0.62	0.68
Asparagine	6	0.49	0.40
Threonine	7	0.88	0.74

* A small amount of methionine is oxidized to the sulfoxide during chromatography with the second solvent.

TABLE I—(*Continued*)

Compound	No.	$R_{proline}$	
		Solvent 1 Ethanol–*tert*.-butyl alcohol–ammonia–water	Solvent 2 *tert*.-Butyl alcohol–formic acid–water
Alanine	8	0.90	1.01
Glutamine	9	0.50	0.56
α-Amino-*n*-butyric acid	10	1.06	1.13
Histidine	11	0.64	0.34
Lysine	12	0.41	0.50
Arginine	13	0.43	0.47
Proline	15	1.00	1.00
Valine	16	1.22	1.22
Leucine	18	1.37	1.29
Isoleucine	18 a	1.42	1.30
Phenylalanine	19	1.33	1.21
Tryptophan	20	1.05	1.06
Tyrosine	21	0.98	0.95
β-Alanine	22	0.65	0.90
γ-Aminobutyric acid	23	0.73	1.00
Hydroxyproline	24	0.77	0.68
Ornithine	102	0.44	0.26
α-ε-Diaminopimelic acid	103	0.16	0.22
2,4-Diaminobutyric acid	106	0.76	0.22
Homoserine	111	0.81	0.77
Canavanine	114	0.32	0.39
Glucosamine	116	1.00	0.46
Putrescine	117	1.10	0.42
Citrulline	118	0.53	0.56
Sarcosine	119	0.88	0.86
Norvaline	125	1.28	1.32
Pipecolic acid	131	1.08	1.21
Histamine	133	1.52	0.53
Tryptamine	137	1.51	1.32
Tyramine	138	2.02	1.17
Cadavarine	161	1.32	0.72
Glutamic acid-monoethyl ester**	162	1.25	1.27
allo-Hydroxylysine	163	0.40	0.25

** Tentatively identified as the gamma-ester. This compound is produced as an artifact of reaction of glutamic acid on a cation exchange resin imbedded in 80 per cent ethanol.

c) *Ninhydrin* (2, p. 123). Ninhydrin in acetone, 0.25 per cent (w/v) containing 1 per cent (v/v) collidine is satisfactory. If *N*-ethylmaleimide has been used, the alkali is neutralized with 1 per cent (v/v) alcoholic acetic acid. The sensitivity of the ninhydrin reaction is decreased after development in *N*-ethylmaleimide.

Table I lists the location of 37 sulfur and 39 nonsulfur compounds in relation to proline. The numbers assigned to the nonsulfur compounds follow the scheme of Steward *et al.* (6). Figure 1 presents a chart for easy reference and location of the positions of individual compounds.

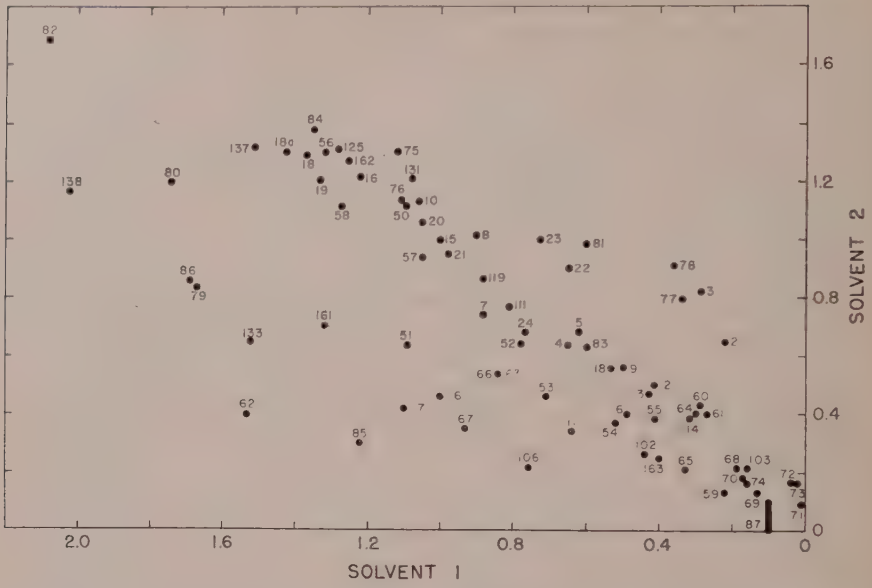

FIGURE 1. Distribution of sulfur-containing amino acids and other compounds on a two-dimensional paper chromatogram. For identification of compounds indicated by the numbers, see Table I.

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